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BIOHYDROGENATION OF LINOLEIC AND ALPHA-LINOLENIC ACID IN RUMEN FLUID *IN VITRO*

DOCTORAL THESIS

Anne Honkanen

DOCTORAL DISSERTATION

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Supervisors: **Professor Aila Vanhatalo**
Department of Agricultural Sciences
University of Helsinki, Finland

Professor Kevin J. Shingfield
Natural Resources Institute Finland (Luke)
Nutritional Physiology, Finland

Reviewers: **Dr Kirsty Kliem**
School of Agriculture, Policy and Development,
Sustainable Agriculture and Food Systems Division,
University of Reading, UK

Dr Annabelle Meynadier
Génétique Physiologie et Systèmes d'Elevag, GenPhySE
Institut National de la Recherche Agronomique (INRA)
Université de Toulouse, France

Opponent: **Dr Susana Alves**
CIISA-Centro de Investigação Interdisciplinar em Sanidade Animal,
Faculdade de Medicina Veterinária,
Universidade de Lisboa, Portugal

Custos: **Professor Aila Vanhatalo**
Department of Agricultural Sciences
University of Helsinki, Finland

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"To make the familiar unfamiliar is what makes learning exciting, stimulates the mind and leads to the ultimate questions."

(Jack Priestley)

ABSTRACT

Some bioactive fatty acid isomers, formed as biohydrogenation (BH) intermediate products in rumen or desaturation products in mammary gland, may confer potential benefits to long-term human health. The objective of the research described in this thesis was to characterize the formation of BH intermediates during incubations of linoleic acid (LeA) and α-linolenic acid (LnA) with rumen fluid, with the potential to identify new BH intermediates and the mechanisms of the BH pathways in rumen. Emphasis was placed on the unknown BH intermediate products. Experiments encompassed *in vitro* trials with pure fatty acids in rumen fluid.

The experiment documented in publication I involved a detailed *in vitro* study in which the effects of the incremental amount of LeA (*cis*-9,*cis*-12 18:2) on disappearance of LeA, formation of BH intermediate products, and the rates of two BH pathways were investigated. The fatty acid composition was determined using complimentary silver-ion thin-layer chromatography, gas chromatography mass-spectrometry, and silver-ion high-performance liquid chromatography. Incubation of incremental amounts of LeA resulted in a dose- and time-dependent accumulation of geometric isomers of Δ9,11 conjugated linoleic acid (CLA) and Δ10,12 CLA, a wide range non-conjugated (NC) 18:2, *cis* 18:1, and *trans* 18:1 intermediate products. Several novel intermediates, including NC *cis*-7,*cis*-12 18:2 and *cis*-8,*cis*-12 18:2, were found to accumulate in direct relation to the amount of added LeA, providing the first indications that hydrogenation of LeA by ruminal bacteria may also involve mechanisms other than hydrogen abstraction or isomerization of the *cis*-12 double bond. Reduction of 18:1 and 18:2 intermediates occurred at much lower rates compared with the formation of CLA and NC 18:2 isomers. At the lowest doses, the end-product was 18:0. The highest amount of LeA inhibited the complete reduction of LeA to 18:0.

The mechanisms of the formation of BH intermediates of LnA (*cis*-9,*cis*-12,*cis*-15 18:3) and identification of new BH intermediates were reported in publication II. LnA was incubated with bovine rumen fluid in buffer prepared using D_2O. *Cis*-9,*trans*-11,*cis*-15 conjugated linolenic acid (CLnA), *trans*-11,*cis*-15 18:2, and *trans*-11 18:1 were the main intermediates with 18:0 as an end-product. Isomers of NC 18:3, such as *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3, and NC isomers of 18:2, such as *cis*-12,*cis*-15 18:2 and *trans*-10,*cis*-15 18:2, and minor amounts of *trans*-9,*trans*-11,*cis*-15 CLnA, Δ9,11,13 CLnA, *trans*-11,*cis*-13 CLA, *trans*-11,*trans*-13 CLA, and *trans*-12,*trans*-14 CLA were formed. The major LnA BH pathway involves the isomerisation to yield *cis*-9,*trans*-11,*cis*-15 CLnA by a mechanism that resembles the formation of Δ9,11 CLA from LeA, indicating ^2H labeling at C-13. NC 18:3 isomers did not differ from natural enrichment, indicating that formation of these products does not involve H exchange with water. Enrichment in n+2 isotopomers of

trans-11,*cis*-15 18:2 was detected due to ^{2}H labelling at C-9 and C-13. The locations of the two ^{2}H labels in the *cis*-12,*cis*-15 18:2 and *trans*-10,*cis*-15 18:2 could not be detected.

Effects of the incremental amount of LnA on disappearance of LnA, formation of BH intermediate products, and the rates of BH were examined in publication III. Incubation of incremental amounts of LnA resulted in a time- and dose-dependent accumulation of geometric isomers of Δ9,11,15 CLnA, and NC 18:2 such as *trans*-10,*cis*-15 18:2 and *trans*-11,*cis*-15 18:2. The unusual intermediates including NC *cis*-7,*cis*-12,*cis*-15 18:3, *cis*-8,*cis*-12,*cis*-15 18:3, and *cis*-12,*cis*-15 18:2, and conjugated CLnA products, such as Δ9,11,13 CLnA, and CLA isomers, including *trans*-11,*trans*-13 CLA and *trans*-12,*trans*-14 CLA were found to accumulate in direct relation to the amount of added LnA. *Trans*-11, *trans*-13/14, -15, -16 18:1, and *cis*-15 18:1 were the main 18:1 products detected. Amount of 18:0 as an end-product did not increase in response to LnA addition. Reduction of 18:1 and 18:2 intermediates occurred at lower rates than the formation of 18:2 isomers from 18:3 isomers. All transfer rates decreased by increases in LnA addition.

The present doctoral thesis work investigated the structure of the major and minor fatty acid isomers formed from LeA and LnA, some of which have not been characterized previously, and possible mechanisms involved in the initial stages of LnA BH by rumen microbiota. The novel isomers identified in this thesis have been used to update the BH pathways for LeA and LnA. These findings explain the appearance of several bioactive fatty acids in muscle and milk that influence the nutritional value of ruminant-derived foods.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Honkanen, A.M., Griinari, J.M., Vanhatalo, A., Ahvenjärvi, S., Toivonen, V. & Shingfield. K.J. 2012. Characterization of the disappearance and formation of biohydrogenation intermediates during incubations of linoleic acid with rumen fluid *in vitro*. *Journal of Dairy Science* 95**:** 1376-1394.

II Honkanen, A.M., Leskinen, H., Toivonen, V., McKain, N., Wallace, R.J. & Shingfield, K.J. 2016. Metabolism of α -linolenic acid during incubations with strained bovine rumen contents: products and mechanisms. *British Journal of Nutrition* 115: 2093–210.

III Honkanen, A.M., Leskinen, H., Vanhatalo, A. & Shingfield, K.J. Disappearance and formation of biohydrogenation intermediates during incubations of incremental dose of α-linolenic acid with mixed bovine rumen microorganisms. (Manuscript).

The publications are referred to in the text by their roman numerals.

CONTRIBUTIONS

The contributions of all authors to the original publications of this thesis are described in the following table (initials of authors are listed in alphabetical order).

	Publications		
Phase of work	I	II	III
Planning the experiment	AH, MG	AH, KS	AH, KS
Conducting the experiment	AH	AH	AH
Laboratory analysis	AH, VT	AH, HL, JW, NM, VT	AH, HL
Data analysis	AH, KS, SA	AH, JW, KS	AH, KS
Drafting the first version of the manuscript	AH	AH	AH
Manuscript preparation	AH, AV, KS, MG, VT	AH, HL, JW, KS, NM	AH, AV, HL

AH = Anne Honkanen
AV = Aila Vanhatalo
HL = Heidi Leskinen
JW = R. John Wallace
KS = Kevin J. Shingfield
MG = J. Mikko Griinari
NM = Nest McKain
SA = Seppo Ahvenjärvi
VT = Vesa Toivonen

ABBREVIATIONS

^{2}H	deuterium, heavy hydrogen, a stable isotope of hydrogen
Ag^{+}-HPLC	silver-ion high-performance liquid chromatography
Ag^{+}-TLC	argentation silver-ion thin-layer chromatography
BH	biohydrogenation
CLA	conjugated linoleic acid, 18 carbon atoms and 2 double bonds
CLnA	conjugated linolenic acid, 18 carbon atoms and 3 double bonds
D	deuterium, heavy hydrogen, a stable isotope of hydrogen
D_2O	deuterium oxide, heavy water, ^{2}H-labelled water
DHA	docosahexaenoic acid
DMOX	4,4-dimethyloxazoline
EPA	eicosapentaenoic acid
FAME	fatty acid methyl esters
GC	gas chromatography
GC-FID	gas chromatography equipped with a flame-ionisation detector
GC-MS	gas chromatography - mass spectrometry
GC-MS/MS	gas chromatography - tandem mass spectrometry
LeA	linoleic acid, *cis*-9,*cis*-12 18:2
LnA	α-linolenic acid, *cis*-9,*cis*-12,*cis*-15 18:3
MPE	moles per cent excess
MUFA	monounsaturated fatty acids
NC	non-conjugated
NEFA	non-esterified fatty acids
NMR	nuclear magnetic resonance
PUFA	polyunsaturated fatty acids
SEM	standard error of the mean
SPE	solid-phase extraction

1 INTRODUCTION

1.1 LIPID METABOLISM IN RUMEN

Ruminants are herbivores, eating plant lipids from forage and concentrate feed containing mainly unsaturated fatty acids. Linoleic acid (LeA) and α-linolenic acid (LnA) are the most abundant fatty acids in ruminant diet containing a 60:40 grass silage to concentrate ratio (Halmemies-Beauchet-Filleau et al., 2013a). Plant oil supplementation such as rapeseed oil, rich in oleic acid, is commonly used to improve meat or milk fatty acid profile. Safflower, sunflower, and soybean oils contain high amounts of LeA, while e.g. linseed and camelina oils are rich in LnA (Zubr, 2003, Giakoumis, 2018). However, ruminant-derived milk and meat contain mainly saturated fatty acids, monounsaturated oleic acid, small amounts of polyunsaturated fatty acid (PUFA), and isomers with *trans* double bonds (Jensen, 2002, Plourde et al., 2007, Shingfield et al., 2008a, Halmemies-Beauchet-Filleau et al., 2013b).

On entering the rumen, dietary lipids are exposed to microbial lipase enzymes and the free fatty acids liberated are subject to biohydrogenation (BH). During the BH, the unsaturated dietary plant fatty acids are converted stepwise to other fatty acid isomers, more saturated isomers, and finally to saturated animal fat.

1.2 RUMINANT-DERIVED BIOACTIVE LIPIDS AND HUMAN HEALTH

Clinical, biomedical, and laboratory studies have provided evidence to suggest that some conjugated linoleic acid (CLA) isomers, formed as BH intermediate products in rumen or desaturation products in mammary gland, may confer potential benefits to long-term human health, including cancer prevention, reduced atherosclerosis risk, improved immune response, potent anti-inflammatory and anti-obesity activities, and improve biomarkers of cardiovascular health (Wahle et al., 2004, Shingfield et al., 2008b). The most abundant CLA isomer in ruminant fat is *cis*-9,*trans*-11 CLA, also known as rumenic acid. Further investigations have indicated that isomers of conjugated linolenic acid (CLnA) have similar biological effects (Koba et al., 2007, Hennessy et al., 2011), with potential in the regulation of blood glucose and body composition in humans (Bassaganya-Riera et al., 2011). *In vitro* studies have demonstrated that CLnA isomers containing a conjugated triene system have higher cytotoxic activity during incubations with human tumour cells than their non-conjugated (NC) counterparts or CLA isomers (Igarashi and Miyazawa, 2000).

In a recent review article, Guillocheau et al. (2019) defined the 'natural dietary *trans* fatty acids' as the n-7 *trans* fatty acid family, including *trans*-9 16:1, *cis*-9,*trans*-11 CLA, and *trans*-11 18:1, naturally occurring in ruminant-derived foods (milk and ruminant meat). They stated that 'because natural *trans* fatty acids are part of the *trans* fatty acid family, they have been compared for decades to their industrial counterparts on a cardiovascular outcome's basis. At current dietary intakes, it is now well recognized that natural *trans* fatty acids are neutral towards cardiovascular health'. Furthermore, Guillocheau et al. (2019) listed the benefits of the natural dietary *trans* fatty acids towards inflammation, obesity, and type 2 diabetes.

1.3 STRUCTURE OF FATTY ACIDS

The LIPID MAPS (http://www.lipidmaps.org) websites provide online resources for an overview of fatty acids and their structures. Fatty acids consist of a carboxylic acid group and a hydrophobic chain consisting of carbon and hydrogen atoms. The chemical formulas of LeA and LnA are $C_{18}H_{32}O_2$ and $C_{18}H_{30}O_2$, respectively. Most of dietary fatty acids have an even number of carbons as part of their structure. Ruminant milk and meat contain also small quantities of fatty acids with an odd number of carbons and fatty acids with branched carbon chain that are derived during microbial metabolism. Ruminant fat contains also keto (oxo), hydroxy, and cyclic fatty acids (Jensen, 2002).

The carbon chain of a saturated fatty acid contains only single bonds, no double bonds. Monounsaturated fatty acids (MUFA) contain one double bond. PUFA contain two or more double bonds. The double bonds between the carbon atoms in the carbon chains of the unsaturated plant lipids contain predominantly double bonds of *cis* configuration, i.e., the hydrogen atoms are on the same side of the double bond in the carbon chain. Ruminant fat and chemically hydrogenated fats (e.g. margarine) may contain *trans* fatty acids, i.e., the hydrogen atoms are on opposite side of the double bond (Shingfield et al., 2008a). *Cis* double bond causes the carbon chain to bend, whereas the carbon chains containing single bonds and *trans* double bonds are straight (Fessenden and Fessenden, 1995). Increasing the number of *cis* double bonds lowers the melting point of fatty acids (liquid at room temperature), whereas saturated fatty acids and fatty acids containing *trans* double bonds are solid at room temperature (Fessenden and Fessenden, 1995).

Methylene interrupted fatty acids, such as LeA and LnA, have a double bond structure with two or more double bonds separated by a single methylene group (-C=C-C-C=C-). Bis-methylene interrupted fatty acids contain two double bonds separated by two methylene groups (-C=C-C-C-C=C-). Conjugated fatty acid is a generic term used for fatty acids with conjugated double bond systems. CLA contains 18 carbon atoms and two double bonds which are separated by one single bond (-C=C-C=C-). CLnA contains 18

carbon atoms and 3 double bonds. Isomers of CLnA containing a conjugated diene double-bond system and one isolated double bond, for example *cis*-9,*trans*-11,*cis*-15 18:3, have similar double bond structure than CLA with one isolated double bond later in the carbon chain (-C=C-C=C-C-C-C=C-). In isomers of CLnA with a conjugated triene system, for example *trans*-9,*trans*-11,*cis*-13 18:3, all three double bonds are separated by one single bond (-C=C-C=C-C=C-).

Ruminant fat is relatively abundant in CLA and CLnA isomers containing a conjugated diene arrangement of double bonds (Plourde et al., 2007, Gómez-Cortés et al., 2009, Lerch et al., 2012a), but only trace amounts of CLnA isomers containing a conjugated triene double bond system, which are typically found in certain plant seeds including pomegranate, tung, bitter gourd, catalpa, and pot marigold (Hopkins and Chisholm, 1968).

1.4 NOMENCLATURE OF FATTY ACIDS

Octadecadienoic acids are fatty acids with 18 carbon atoms and two double bonds (IUPAC-IUB, 1977). Fatty acids containing 18 carbon atoms and three double bonds are called octadecatrienoic acids. 'C:D' is the ratio of the total amount of carbon atoms of the fatty acid in relation to the number of double bonds in it. The 'C' stands for carbon atoms and the 'D' stands for double bond. For example, 18:2 is a fatty acid with 18 carbons and 2 double bonds. The 18:3 is a fatty acid with 18 carbons and 3 double bonds.

The position of the double bonds in the carbon chain can be described using Δ, omega or n in the name. The 'omega' or 'n' describes the double bond position by counting the number of double bonds from the methyl end (n-terminus) of the fatty acid. Omega-3 (ω-3 or n-3) and omega-6 (ω-6 or n-6) polyunsaturated fatty acids have the last double bond 3 or 6 carbons from the methyl end of the carbon chain, respectively (for example 18:2 n-6 or 18:3 n-3). The delta (Δ) nomenclature identifies a fatty acid by counting the position of a double bond from the carboxylic side of the fatty acid. If known, the geometric configuration of each double bond can be expressed as a *cis* (or Z) or *trans* (or E), for example *cis*-9, *cis*-12 18:2 (or 9Z12Z 18:2) (IUPAC-IUB, 1977).

Some fatty acid isomers are better known by their trivial names (IUPAC-IUB, 1977). For example, *cis*-9,*cis*-12 18:2 (18:2 n-6 or 18:2 ω-6) is called LeA. *Cis*-9,*cis*-12,*cis*-15 18:3 (18:3 n-3 or 18:3 ω-3) is called LnA. *Cis*-9 18:1 is oleic acid. Stearic acid is a saturated fatty acid containing 18 carbon atoms and no double bonds (18:0). *Trans*-11 18:1 is vaccenic acid, *cis*-9,*trans*-11 CLA is rumenic acid, and *cis*-9,*trans*-11,*cis*-15 CLnA is rumelenic acid (Destaillats et al., 2005).

1.5 PARTIAL HYDROGENATION AND BIOHYDROGENATION

The partial hydrogenation is the process of converting unsaturated fatty acids into the more saturated fatty acids. This can be performed by chemical hydrogenation in industry or by microbial BH in living organisms (Menaa et al., 2013). The partial hydrogenation of plant oils produces various hydrogenated fatty acids, including several isomers of octadecenoic acids (18:1), depending on the reaction conditions.

In contrast, microbial BH is catalysed by enzymes, such as Δ^{12}-*cis*,Δ^{11}-*trans* isomerase (Kepler and Tove, 1967), Δ-9 isomerase (Deng et al., 2007, Farmani et al., 2010), and *cis*-9,*trans*-11 CLA reductase (Hughes et al., 1982, Fukuda et al., 2007). The enzymes can selectively convert LeA and LnA to specific fatty acid isomers (Kepler et al., 1966, Wilde and Dawson, 1966, Harfoot and Hazlewood, 1997, Enjalbert et al., 2017). Isomerase enzyme isolated from *P. acnes* is capable of converting LeA to *trans*-10,*cis*-12 CLA (Deng et al., 2007) and LnA to CLnA isomers with *trans*-11,*trans*-13,*cis*-15 CLnA as the main product with trace amounts of *trans*-10,*cis*-12,*cis*-15 CLnA also being formed (Hornung et al., 2005).

The major pathway of LeA BH involves isomerization to form *cis*-9,*trans*-11 CLA followed by reduction to *trans*-11 18:1 and finally to 18:0 (Harfoot and Hazlewood, 1997). A minor pathway of LeA BH involves the formation of *trans*-10,*cis*-12 CLA which is reduced to *trans*-10 18:1 and finally to 18:0 (Griinari and Bauman, 1999, Wallace et al., 2007).

BH of LnA in the rumen has been described by a metabolic pathway that involves an initial isomerization to a conjugated 18:3 (*cis*-9,*trans*-11,*cis*-15 CLnA) followed by sequential reduction of double bonds at 9, 15, and 11 to yield *trans*-11,*cis*-15 18:2, *trans*-11 18:1, and 18:0 (Wilde and Dawson, 1966). Several putative pathways, involving the formation of *trans*-9,*trans*-11,*cis*-15 CLnA (Waşowska et al., 2006), *cis*-9,*trans*-13,*cis*-15 18:3 (Destaillats et al., 2005), and *trans*-10,*cis*-12,*cis*-15 CLnA (Griinari and Bauman, 1999) as initial intermediates, have also been proposed, but not substantiated.

Decreases in pH (Troegeler-Meynadier et al., 2003, Fuentes et al., 2009), high starch diets and addition of LeA (Zened et al., 2012) or dietary supplementation of DHA-enriched supplements (Dewanckele et al., 2018) could induce a shift from the main BH pathway toward the formation of *trans*-10 intermediates, e.g., *trans*-10,*cis*-15 18:2, and *trans*-10 18:1. Any significant increase of *trans*-10 isomers at the expense of *trans*-11 isomers can be considered as a shift (Zened et al., 2012).

1.6 BACTERIA CAPABLE OF BIOHYDROGENATION

Rumen fluid is a mixed culture of anaerobic micro-organisms, such as bacteria (10^{10}/g digesta), ciliate protozoa (10^{6}/g digesta), anaerobic fungi (10^{3} spores/g digesta), and methanogenic archaea (10^{8}/g digesta) (Fonty et al., 1987, Nagaraja, 2016). Bacteria, rather than protozoa and fungi, are thought to be responsible for BH (Enjalbert et al., 2017).

Rumen microbial community composition varies with diet and host animal, but a core microbiome is found across a wide geographical range (Henderson et al., 2015). Recent results suggest a number of pathways for LnA BH are operating concurrently in the rumen, with their balance being influenced by diet and driven by less abundant species rather than members of the core bacterial population (Petri et al., 2018). Relatively few strains of bacteria capable of BH have been identified (Harfoot and Hazlewood, 1997, Lourenço et al., 2010, Enjalbert et al., 2017). *Butyrivibrio fibrisolvens* (Kepler et al., 1966), *Butyrivibrio hungatei* (Van de Vossenberg and Joblin, 2003), *Butyrivibrio proteoclasticus* (formerly *Clostridium proteoclasticum*, Wallace et al., 2006) (Moon et al., 2008), *Propionibacterium acnes* (Wallace et al., 2006), *Lactobacillus plantarum* (Ogawa et al., 2005, Kishino et al., 2009, 2010, 2013), *Bifidobacterium breve* (Coakley et al., 2003), and *Pseudobutyrivibrio* isolates (Paillard et al., 2007) are known to be capable to hydrogenate LeA or LnA or both. However, the ruminal bacteria involved in *trans*-10 formation remain unclear. In some studies, *P. acnes* has been shown to convert LeA to *trans*-10,*cis*-12 CLA (Deng et al., 2007, Wallace et al., 2007, McKain et al., 2010), but not in all studies (Dewanckele et al., 2018).

Butyrivibrio hungatei (Paillard et al., 2007) and *Butyrivibrio proteoclasticus* (Li et al., 2012) participate in the conversion of *trans*-11 18:1 to 18:0, the last step of BH. High concentrations of LeA are known to inhibit microbial growth, depending on bacterial species, and arrest the hydrogenation of unsaturated fatty acids (Polan et al., 1964, Harfoot et al., 1973a, Maia et al., 2007). *B. hungatei* and *B. proteoclasticus* are more sensitive to the toxic effects of PUFA than other *Butyrivibrio* or *Pseudobutyrivibrio* species (Wallace et al., 2006, Paillard et al., 2007). As only these two bacterial species are known to be responsible for this last step of BH, the step is rate limiting.

1.7 MECHANISMS OF *CIS*-12 TO *TRANS*-11 ISOMERISATION

The first step of the major BH pathways of LeA and LnA is the isomerisation of *cis*-12 double bond to *trans*-11. A Δ^{12}-cis,Δ^{11}-trans isomerase enzyme (linoleate isomerase, EC 5.2.1.5) converts *cis*-12 double bond to *trans*-11 double bond. Kepler et al. (1971) demonstrated that the enzyme isolated from

B. fibrisolvens strain A38 (Kepler and Towe, 1967) or *Treponema* (Yokoyama and Davis, 1971) is specific for the *cis*-9,*cis*-12 structure and requires a free carboxyl group. Kepler et al (1971) proposed a mechanism for the interaction between LeA substrate and the hydrophobic binding site of the Δ^{12}-*cis*,Δ^{11}-*trans* isomerase enzyme. The active centre of the enzyme is hydrophobic, and it consists of three active regions. The electronegative region of the enzyme acts as a hydrogen binding site and binds the free carboxyl group of the fatty acid to the enzyme by hydrogen linkage. It also absorbs a proton from the 11th carbon of the LeA or LnA. The second region donates a proton to the 13th carbon of the LeA or LnA. The third region is responsible for interaction between the active site of the enzyme and the π electrons of the double bond at *cis*-9. Although the Δ^{12}-*cis*,Δ^{11}-*trans* isomerase is specific for the *cis*-9,*cis*-12 structure, the presence of *cis*-15 double bond does not seem to affect the enzyme activity (Kepler and Tove, 1967, Yokoyama and Davis, 1971).

1.8 STATE OF THE ART

Numerous *in vitro* and *in vivo* studies have allowed the major pathways of LeA and LnA BH in the rumen to be elucidated (Wilde and Dawson, 1966, Kellens et al., 1986, Harfoot and Hazlewood, 1997), but limited information is available on the formation of other intermediates (Griinari and Bauman, 1999, Troegeler-Meynadier et al., 2003, Destaillats et al., 2005, Waşowska et al., 2006, Jouany et al., 2007, Wallace et al., 2007). The known, major and minor, and presumptive BH pathways of LeA and LnA in rumen have been summarised in recent review articles (Białek et al., 2017,Ferlay et al., 2017).

In a review, Jenkins et al. (2008) concluded that the desire to focus on major intermediates and avoid complications has led to the persistence of many oversimplified pathways of ruminal BH over the decades. Jenkins et al. (2008) speculated that *cis*-9,*trans*-11 CLA probably was the only CLA isomer that could be detected in many older chromatographic techniques. Thus, *cis*-9, *trans*-11 CLA persisted over time as the only CLA isomer seen in pathways of BH.

The availability of longer columns (100 m or more) for gas chromatography (GC) combined with new techniques in mass spectroscopy (MS) have enabled separation and identification of minor CLA isomers and other BH products. Incubations of ^{13}C-labelled LnA with bovine rumen contents were reported to result in the accumulation of 14 uncharacterised 18:3 intermediates, but the position and geometry of double bonds could not be identified (Lee and Jenkins, 2011). Nowadays, a total of 20 CLA isomers have been identified in bovine milk fat, with geometrical configuration of *cis*,*cis*, *cis*,*trans*, *trans*,*cis,* and *trans*,*trans*, and the double bond position from carbons 6,8 to 13,15 (Halmemies-Beauchet-Filleau et al., 2013b). A total of 6 CLnA isomers have

been identified from milk fat (Destaillats et al., 2005, Lerch et al., 2012a) or rumen content (Waşowska et al., 2006).

The proper identification of BH intermediates of unsaturated fatty acids is a great analytical challenge, mainly due to the relatively small concentrations of these compounds in assayed samples, lack of their standards, and reference mass spectra, frequent co-elution as well as poor resolution (Alves and Bessa, 2014). The most important task in analysis of conjugated fatty acids is to establish the position of double bond and its geometrical configuration (*cis* or *trans*) in the carbon chain (Białek et al., 2017).

Even though several studies have characterised a range of intermediates formed during the incubation of LnA with digesta collected from the ovine or bovine rumen (Waşowska et al., 2006, Jouany et al., 2007, Lee and Jenkins, 2011), none have provided evidence on the mechanisms responsible. A more complete and detailed understanding of the mechanisms responsible for the formation of CLA isomers from LeA and the formation of CLA and CLnA isomers from LnA could be crucially important for artificial synthesis of these compounds for therapeutic use in the prevention of human chronic diseases or characterising activity in a range of mammalian species, including ruminants.

2 OBJECTIVES AND HYPOTHESES OF THE THESIS

The present *in vitro* studies investigated BH of LeA (I) and LnA (II, III) during incubations with bovine rumen fluid. Formation of fatty acid intermediates over time was determined to provide a detailed and comprehensive assessment of LeA (I) and LnA (II, III) metabolism. Possible mechanisms responsible for the formation of fatty acid intermediates were investigated by examining the incorporation of ^{2}H (deuterium) in products during incubations of LnA and buffer prepared using ^{2}H-labelled water (deuterium oxide, D_2O) (II). The aims were to discern possible fatty acid isomers formed in the rumen, to provide evidence for the major and minor BH pathways, and to explain the appearance and origin of several bioactive fatty acids in ruminant-derived meat and milk.

The main hypotheses tested in this research were:

1) Increases in the initial dose or LeA and LnA decreases the disappearance of LeA and LnA and increases the formation of BH intermediates over time during incubations with strained bovine rumen fluid (I, III)
2) Several CLnA isomers previously identified in ruminant milk and muscle fat originate from BH of LnA in rumen (II, III).
3) Several CLA isomers are formed during BH of LeA and LnA in rumen fluid (I-III).
4) *Cis*-12 18:1 and *cis*-12,*cis*-15 18:2, previously identified in ruminant milk and muscle fat, are formed in rumen during BH of LeA and LnA, respectively (I-III).
5) The initial steps of BH of LeA and LnA also involve migration of the *cis*-9 double bond rather than isomerization of the *cis*-12 double bond (I-III).
6) *Cis*-9,*trans*-11,*cis*-15 18:3 is formed from LnA via mechanism identical to that responsible for the conversation of LeA to geometric isomers of Δ9,11 CLA (II).
7) Δ10,12,15 18:3 and *trans*-10,*cis*-15 18:2 are the missing intermediates of *trans*-10 shifted rumen BH pathway of LnA (II, III).

3 MATERIALS AND METHODS

In vitro incubations were conducted at University of Helsinki, Finland (I) and at MTT Agrifood Research Finland (Natural Resources Institute Finland (Luke) since 2015), Jokioinen (II, III). Fatty acid analyses were conducted at University of Helsinki (I) and at Luke (I-III). Experimental procedures have been reported in detail in the original publications (I-III).

3.1 *IN VITRO* INCUBATIONS

The studies documented in I-III were conducted as three separate *in vitro* experiments (Table 1). In experiment I, rumen fluid was collected 1 h after morning feeding from a multiparous nonlactating Finnish Ayrshire dairy cow. In experiments II and III, the rumen fluid was collected immediately before morning feeding from four cows in late lactation in order to obtain a more diverse microbial population. All cows were fitted with rumen cannulae. The cows received a diet based on a mixture of timothy and meadow fescue grass silage and concentrates, forage:concentrate ratio 70:3 (I) or 60:40 (II, III), on a dry matter basis. Rumen fluid was strained through cheesecloth, and mixed (1:2, vol/vol) with warm (39°C), degassed (CO_2) modified McDougall's buffer (McDougall, 1948). In experiments I and III, the buffer was prepared using deionised water (H_2O). In experiment II, the buffer was prepared using deuterium oxide (D_2O).

LeA (*cis*-9,*cis*-12 18:2) (I) or LnA (*cis*-9,*cis*-12,*cis*-15 18:3) (II, III) was incubated as an oil-in-water suspension (Rainio et al., 2001) with strained rumen fluid. Suspensions were prepared daily, immediately before incubations, by mixing deionised water with Tween 80 and LeA or LnA, followed by the addition of NaOH. Once prepared, suspensions of LeA or LnA were diluted with deionized water. Amounts of LeA or LnA, Tween 80, and NaOH added to each flask were adjusted to be in the same ratio.

In the experiment I, four stock solutions of LeA suspensions resulting, 1.0, 2.5, 5.0, and 10 mg of LeA/flask, were prepared. In the experiment II, the amount of 5 mg of LnA/flask was used. In the experiment III, four stock solutions of LnA suspensions, resulting 2.5, 5.0, 7.5 mg, and 10 mg of LnA/flask, were prepared.

LeA or LnA stock emulsions (2.5 ml), 50 ml of buffered ruminal fluid, 400 mg of finely ground dry grass hay, and nonadecanoic as an internal standard (19:0, 5 mg/ml in ethanol) were transferred into 100-ml glass flasks. Ground hay was included as a fermentation substrate, because anaerobes do not generate ATP from LeA or LnA (Fievez et al., 2007), and also to provide a site

for attachment, as the majority of BH occurs on the surface of feed particles (Harfoot et al., 1973b). Flasks containing rumen fluid and all reagents except added LeA or LnA stock emulsions were also prepared and used as control samples. Each flask was degassed with CO_2, incubated on a benchtop shaker, and maintained in the dark at 39°C for 0, 1.5, 3.0, 4.5, 6.0, and 9.0 h (I), 0, 1.5, 3.0, 12 h (II) or 0, 1.5, 3.0, 6.0, and 12.0 h (III). Incubations of LeA or LnA were limited up to 9 h and 12 h, respectively, to avoid potential losses in the activity of microbes in incubation flasks (Jouany et al., 2007). The incubation time of LnA was extended from 9 h to 12 h to obtain more BH end products. At the end of each designated time point, incubations were stopped by placing the flasks into ice-cold water. Gas was not vented from the flasks during the incubations. The pH of flask contents was measured. The contents of each flask were frozen (−20°C), freeze-dried, and stored at −20°C until fatty acid determinations.

Incubations were repeated over 5 separate days (I) or 3 days (III) with 1 flask for each incubation time per treatment per day (n = 5 or 3, respectively). In the experiment II, incubations were performed in triplicate using rumen fluid from each of the four donor cows separately (n = 12).

Table 1. The overview of the experiment design, objectives, and methods (I-III)

Publ.	Fatty acid[1]	Fatty acid dose, incubation time, days, replicates, cows	Objectives	Methods[2]
I	LeA	1.0, 2.5, 5.0, and 10 mg of LeA/flask 0, 1.5, 3.0, 4.5, 6.0, and 9.0 h 5 days, 1 rep/day, 1 cow	- LeA disappearance -Identification of minor fatty acid isomers - Effect of incremental doses of LeA	Ag^+-HPLC, Ag^+-TLC, GC-FID, GC-MS, SPE, WinSAAM, NLIN SAS, MIXED SAS
II	LnA	5 mg LnA/flask, D_2O 0, 1.5, 3.0, and 12 h 1 day, 3 rep., 4 cows separately	-Identification of minor fatty acid isomers -Biohydrogenation mechanisms	Ag^+-HPLC, GC-FID, GC-MS, deconvolution calculations (for ^2H-enrichment), MIXED SAS
III	LnA	2.5, 5.0, 7.5, and 10 mg of LnA/flask 0, 1.5, 3.0, 6.0, and 12.0 h 3 days, 1 rep/day, 4 cows mixed	-LnA disappearance - Effect of incremental doses of LnA	Ag^+-HPLC, GC-FID, GC-MS, WinSAAM, NLIN SAS, MIXED SAS, POWER SAS

1 LeA, linoleic acid; LnA, α-linolenic acid

2 Ag^+-TLC, argentation silver-ion thin-layer chromatography; Ag^+-HPLC, silver-ion high-performance liquid chromatography; GC-FID, gas chromatography equipped with a flame-ionisation detector; GC-MS, gas chromatography-mass spectrometry; GC-MS/MS, gas chromatography-tandem mass spectrometry; NLIN SAS, nonlinear model procedure of Statistical Analysis System software; MIXED SAS, a mixed linear model procedure of Statistical Analysis System software; POWER SAS, a procedure of of Statistical Analysis System software for power and sample size analyses; SPE, solid-phase extraction; WinSAAM, Windows version of the Simulation, Analysis, and Modeling computer program

3.2 LIPID ANALYSIS

In all experiments, in order to dissociate possible calcium salts and to enhance the transfer of free fatty acids to the solvent, deionized water, and 2 M hydrochloric acid was added to the freeze-dried flask contents until pH was 2.0. Lipid was extracted twice using a mixture (3:2, vol/vol) of hexane (I) and isopropanol or heptane (II, III) and isopropanol. In experiment II and III, the hexane was replaced with heptane as the yield and quality of the oil extracted by heptane has been shown to be similar to that extracted by hexane, but heptane does not have the environmental and health concerns associated with the hexane (Conkerton et al., 1995). Extractions were washed with deionized water, dried over sodium sulfate, and evaporated to dryness at 45°C (I) or, to reduce the possibility of oxidation, at room temperature (II, III) under a constant stream of N_2.

As the LeA was added to the incubation flasks as free fatty acids, NEFA were separated from other lipid fractions by solid-phase extraction (SPE) using aminopropyl-bonded silica cartridges (I). Fatty acid methyl esters (FAME) were prepared by incubations with 1% (vol/vol) sulfuric acid in methanol at 50°C for 30 min. To avoid the rather laborious and unnecessary SPE step in the experiments II and III, FAME derivatives were prepared from total lipid using a 2-step base acid catalysed procedure based on incubations with sodium methoxide in methanol followed by the incubations with sulfuric acid in methanol.

The FAME were fractionated to 1) saturates, 2) *cis*,*trans*, *trans*,*cis*, and *trans*,*trans* 18:2 isomers, and 3) *cis*,*cis* 18:2 isomers using complimentary argentation silver-ion thin-layer chromatography (Ag^+-TLC) (I). The fatty acid isomers were separated and quantified by a gas chromatography equipped with a flame-ionisation detector (GC-FID) and a 100-m fused silica capillary column coated with a film of cyanopropyl polysiloxane (CP-SIL 88). An alternative 100-m column coated with a highly polar ionic liquid SLB-IL111 was used to separate FAME of *trans*-10,*cis*-15 18:2 and *trans*-11,*cis*-15 18:2 (II, III). An Ag^+-HPLC using four silver-impregnated silica columns coupled in series was used to separate and determine the ratio of CLA isomers. Concentrations of specific CLA isomers were calculated based on proportionate peak area responses determined by HPLC and the sum of *trans*-7,*cis*-9 CLA, *trans*-8,*cis*-10 CLA, and *cis*-9,*trans*-11 CLA determined as a single peak during GC analysis.

Samples of FAME were converted to 4,4-dimethyloxazoline (DMOX) derivatives by incubation overnight with 2-amino-2-methyl-1-propanol under a N_2 atmosphere at 170°C.

3.3 IDENTIFICATION OF FATTY ACIDS

Chromatogram peaks were initially identified by comparison of retention times with authentic FAME standards. The structure of the DMOX derivatives, i.e., number of carbon atoms, and number and position of double bonds in the carbon chain, was determined by a GC equipped with a quadrupole selective mass detector (GC-MS), operated in the positive electron ionisation mode.

Interpretation of mass spectra was undertaken according to published guidelines (Spitzer, 1996, Christie, 1998, Christie, 2018). Electron impact ionization spectra of DMOX derivatives were used to locate double bonds based on atomic mass unit distances. An interval of 12 atomic mass units (amu) between the most intense peaks of clusters of ions containing n and n-1 carbon atoms was interpreted as cleavage of the double bond between carbon n and n+1 in the fatty acid moiety. An interval of 14 amu was interpreted as a single bond. Odd-numbered ion fragments at m/z 139, 153, and 167 were used as diagnostic ions to locate double bonds at Δ4, Δ5, and Δ6, respectively (Spitzer, 1996). When available, deduced fatty acid structure was verified by cross-referencing with earlier reports (Spitzer, 1996, Christie, 1998), and with an online reference library of DMOX derivative electron ionization mass spectra (Christie, 2019).

Furthermore, the isomers containing bis-methylene interrupted double bonds were identified based on an ion fraction with high relative intensity between the double bonds. Characteristic ion fragments of all bis-methylene interrupted DMOX derivatives are listed in Table 2.

Table 2. Characteristic ion fragments of bis-methylene interrupted DMOX derivatives.

Fatty acid	Key diagnostic ion fragment (m/z)	Reference
Δ5,9 18:2	180	Christie, 1998
Δ6,10 18:2	194	Nikolova et al., 2006
Δ7,11 18:2	208	Nikolova et al., 2006
Δ8,12 18:2	222	Nikolova et al., 2006
Δ9,13 18:2	236	Nikolova et al., 2006
Δ10,14 18:2	250	Nikolova et al., 2006
Δ11,15 18:2	264	Nikolova et al., 2006
Δ12,16 18:2	278	Nikolova et al., 2006
cis-9,*trans*-11,*cis*-15 CLnA	262	Destaillats et al., 2005

The mass spectra of the DMOX derivatives by GC-MS cannot be used to detect the configuration of the double bond geometry. Double bond geometry of CLnA isomers was deduced based on

1) retention time of FAME authentic standards during GC-FID analysis (a mixture geometric isomers of 8,10,12-CLnA and 9,11,13-CLnA, Larodan Fine Chemicals AB),
2) the known elution order of authentic standards (a mixture of geometric isomers of 9,12 18:2, L-8404; Sigma, St. Louis, MO),
3) by cross-referencing elution order in milk fat, meat, and rumen fluid analyses by GC-MS (Table 3),
4) by cross- referencing elution order in milk fat samples analysed by a GC and covalent adduct chemical ionization tandem MS (GC-MS/MS) in literature (Table 3).

Table 3. Characterization of the double bond geometry of conjugated linolenic acid isomers.

Fatty acid	Source	Identification method	Reference
cis-9,*trans*-11,*trans*-15 CLnA	milk	GC-MS/MS	Gómez-Cortés et al., 2009
	milk	GC-MS	Lerch et al., 2012a
cis-9,*trans*-11,*cis*-15 CLnA	milk	GC-MS	Destaillats et al., 2005
	rumen fluid	GC-MS	Waşowska et al., 2006
	milk	GC-MS/MS	Gómez-Cortés et al., 2009
	milk	GC-MS	Lerch et al., 2012a
cis-9,*trans*-13,*cis*-15 CLnA	milk	GC-MS	Destaillats et al., 2005
	meat	GC-MS, NMR	Plourde et al., 2007
cis-9,*trans*-11,*trans*-13 CLnA	milk	GC-MS	Lerch et al., 2012a
trans-9,*trans*-11,*cis*-15 CLnA	rumen fluid	GC-MS	Waşowska et al., 2006

GC-MS, gas chromatography - mass spectrometry; GC-MS/MS, gas chromatography - tandem mass spectrometry; NMR, nuclear magnetic resonance spectrometer

For FAME not available as authentic standards or references in literature, double bond geometry was inferred from the relative retention time, order of elution during GC-FID analysis (I-III), and by using Ag^+-TLC to fractionate the different double bond geometries (I).

Identification of CLA isomers was performed using commercially available CLA FAME standards, and verified by cross-referencing with the elution order reported in the literature (Delmonte et al., 2005) using *cis*-9,*trans*-11 CLA as a landmark isomer. Isomers of CLnA with a conjugated triene system were identified based on retention time comparisons with FAME standards containing geometric isomers of Δ8,10,12 CLnA and Δ9,11,13 CLnA.

3.4 CALCULATIONS AND STATISTICAL ANALYSIS

The amounts of LeA, LnA, and intermediates that accumulated over time during incubations of LeA or LnA with ruminal fluid were expressed as mg/flask. Concentrations of fatty acids were corrected by subtracting the amount of fatty acids measured in corresponding control samples containing rumen fluid and all reagents except LeA (I) or LnA (III) from the amounts of fatty acids determined for all treatment samples. In the study on LnA incubated with D_2O (II) the amount of control samples was not subtracted. The values were used to calculate the LeA (I) or LnA (III) disappearance, and the rates of BH intermediate and end-product formation. Kinetic parameters of LeA and LnA disappearance were estimated according to a first-order exponential model (Ørskov and McDonald, 1979), defined as

$$Q_t = a + b(1 - e^{-ct})$$

where Q_t represents the percentage of LeA or LnA that disappears in time t (h), expressed as a percentage of the initial amount, a is the disappearance of LeA or LnA at time 0 h (expressed as a percentage of the initial amount), b is the percentage of LeA or LnA that can potentially disappear during incubations with ruminal contents (expressed as the percentage of the initial amount), and c is the fractional rate of fraction b (expressed as 1/h). As the disappearance of LeA and LnA was found to be instantaneous, lag time was not included in the model. Parameters a, b, and c were computed using the NLIN procedure of the SAS (version 9.4, SAS Institute, Inc.).

The optimum sample size was determined by power analysis (III) implemented with POWER procedure of SAS using onewayanova statement. The means and variance estimates used in the power analysis were obtained from the experiment on LeA (I), and from the actual data of the LnA study (III). Two-sided significance level was 0.05. This yielded the statistical power of > 90% which shows that the three samples per incubation time point was enough for the designed purpose in the *in vitro* experiment on incremental dose of LnA.

Least square means of total amounts of LeA, CLA, NC 18:2, 18:1, and 18:0 formed during incubations of LeA (I), and total amounts of 18:3, 18:2 and 18:1 isomers, and 18:0 formed during incubations of LnA (III) with bovine ruminal fluid were fitted to a dynamic multi-compartmental model, the multiple-pool, first-order kinetic model. Fractional rates of transfer among the fatty acid pools were estimated by WinSAAM software (Version 3.2.0, University of Pennsylvania, Philadelphia) (I, III). Mass spectra of FAME were used to calculate the moles per excess (MPE) ratios of fatty acid products formed during incubations of LnA with D_2O, and with de-ionized H_2O (II). Enrichments (MPE incubation product/MPE water) of n + 1, n + 2, and n + 3 isotopomers were calculated from the m/z ratios by deconvolution (Wallace et

al., 2007) (II). As pH is a log scale, the pH values were converted to hydrogen ion concentration prior to statistical analysis, and retro-converted back to pH values before plotting on charts.

The enrichment data, the amounts of fatty acids (mg/flask), pH, and the parameters describing the disappearance of LeA and LnA were analysed by ANOVA for repeated measures using the MIXED linear model procedure of SAS. Denominator degrees of freedom were calculated using the Kenward-Rogers method (II, III). Least square means with pooled standard errors of the mean (SEM) were reported and compared using Tukey-Kramer test (II, III). Treatment effects were considered significant at $P < 0.05$.

4 RESULTS AND DISCUSSION

4.1 *IN VITRO* CONDITIONS AND PH

In the current experiment, an *in vitro* batch culture method was used to examine the fate of LeA or LnA during incubations with strained ruminal fluid over a range of doses that could be expected under physiological conditions. Since high concentrations of LeA are known to inhibit microbial growth, the concentrations of added LeA or LnA and incubation times were selected based on incubations with several doses of LeA and LnA prior to the actual experiments. In this *in vitro* study, the amount of added LeA or LnA in incubation flask varied from 0.02 to 0.2 mg/ml of buffered ruminal fluid. The initial amount of LeA in the hay and rumen fluid in the control flasks without added LeA or LnA was 0.005 mg/ml (0.26 mg /flask, I) and 0.014 mg/ml of buffered ruminal fluid (0.72 mg /flask, II and III). The initial amount of LnA in the hay and rumen fluid in the control flasks was 0.006 mg/ml (0.32 mg /flask, I) and 0.014 mg/ml of buffered ruminal fluid (0.71 mg /flask, II and III). In other *in vitro* studies, the concentration of LeA or LnA varied from 0.04 to 0.5 mg/ml of buffered ruminal fluid (Fievez et al., 2007).

Rumen fluid was strained through 2 layers of cheesecloth. Preliminary investigations in our laboratory confirmed that this procedure ensures that small particles are retained in the rumen inoculum. Because the feed particles are considered the major site of dietary PUFA BH by bacteria in the rumen (Harfoot et al., 1973b), emulsions of LeA or LnA were added directly on top of the ground hay before the addition of rumen fluid to mimic conditions *in vivo*. In a previous *in vitro* study (Troegeler-Meynadier et al., 2014), the disappearances of LeA and formation of BH products were higher with hay than starch substrate as the fibrolytic bacteria, including biohydrogenating ones, were more likely to develop on hay as the fermentation substrate than on starch. The disappearance of LnA was not affected by the feed substrate (Troegeler-Meynadier et al., 2014). In this study, hay was the only source of nitrogen offered to the bacteria.

Potential inhibitory effects of LeA on microbial growth were minimised in this study by dispersing LeA in a sufficient concentration of polyoxyethylene sorbitan monooleate detergent (Tween 80) (Rainio et al, 2001) containing 10% total fatty acids consisting of 71.8% *cis*-9 18:1, and 0.17% LeA in the fatty acid composition (Khiaosa-ard et al., 2010). Alternative methods of introduction of the water-insoluble PUFA into incubation flask are 1) emulsion through sonication, 2) adding fatty acids as PUFA-hexane solution with the hexane being evaporated prior to incubations, 3) dissolving PUFA in ethanol, and 4) use of bovine serum albumin to ensure that fatty acids remain in suspension (Fievez et al., 2007). A comparison reported that emulsions containing LeA prepared using Tween 80, rather than ethanol or sonication, resulted in more

extensive hydrogenation of LeA, greater accumulation of 18:1 intermediates, and higher 18:0 production during incubations with rumen fluid (Khiaosa-ard et al., 2010).

Due to poor dissolution in Tween 80 and water, the internal standard 19:0 was dissolved in small amount of ethanol. As only part of the incubation flask contents was used for the lipid analysis and considering losses of dry matter due to gas production during the incubations, the internal standard was added in the incubation flasks before the incubations. Furthermore, the 19:0 went through all the same laboratory treatments as the LeA or LnA added to the incubation flasks. Therefore, possible losses of the fatty acids during the laboratory treatments should not affect the ratio of the concentrations of the internal standard, LeA or LnA, and fatty acid intermediates formed from the LeA and LnA. The 19:0 was not assumed to metabolise during the incubations.

Low pH (below 6) inhibits Δ^{12}-cis,Δ^{11}-trans isomerase (Kepler and Tove, 1967) and isomerisation of LeA (Troegeler-Meynadier et al., 2006, 2014). During the course of incubations, the pH of incubation flask contents decreased from 6.6 to 6.2 for LeA (Figure 1A, I) and from 6.7 to 6.2 for LnA (Figure 1B, III). The pH of incubation flasks from 0 h to 6 h was not altered by the initial amount of LeA or LnA or due to interactions between the amount of added LeA or LnA and incubation time. However, after 9 h or 12 h incubation, a higher pH was detected with higher doses of added LeA or LnA, indicating possible loss of microbial activity. After 9 h incubation, pH was lower in control flasks and flasks containing 1.0 and 2.5 mg of added LeA (pH 6.19) compared with 10 mg of added LeA (pH 6.30). After 12 h incubation of LnA, pH was lower in control flasks and flasks containing 2.5 mg and 5 mg of added LnA (pH 6.25) compared with 10 mg of added LnA (pH 6.31).

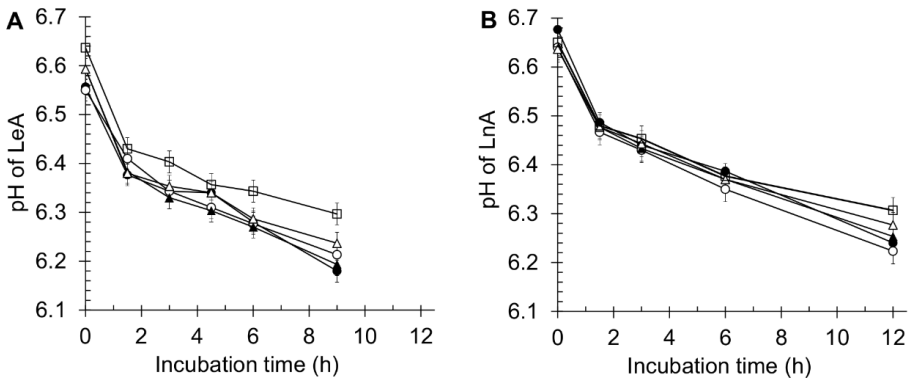

Figure 1 Temporal changes in mean pH of flask contents during incubations of 0.0 (●), 1.0 (○), 2.5 (▲), 5.0 (Δ), and 10.0 (□) mg of LeA (A, I), and 0.0 (●), 2.5 (○), 5.0 (▲), 7.5 (Δ), and 10.0 (□) mg of LnA (B, III) with strained bovine ruminal fluid. Values represent least squares means. SEM = 0.023 and 0.026 for pH of LeA and pH of LnA, respectively.

In vitro conditions affect estimates of rumen BH and their kinetics compared to *in vivo* conditions. Exact values are comparable only within the same *in vitro* conditions. To allow correct interpretation of data on the accumulation of BH intermediates, inclusion of control samples under the same *in vitro* conditions are needed (Fievez et al., 2007). In this study, the incubation media of the control samples, containing rumen fluid, hay, and all reagents without added LeA or LnA, contain small amounts of LeA, LnA, and other fatty acids. In order to detect only the fatty acid intermediates formed from the added LeA or LnA, concentrations of fatty acids in the treatment samples were corrected by subtracting the amount of fatty acids measured in corresponding control samples. However, the added LeA or LnA may decrease the rate of BH of the fatty acids in the incubation media. In some cases, the incubation media of the control flasks may contain even more BH intermediates and end products than the flasks with added LeA or LnA. This causes negative values after subtracting the fatty acids in the control samples from the fatty acids in the treatment samples.

Overall, the *in vitro* method used in this study shows the potential to appropriately simulate BH of LeA and LnA *in vivo* as 1) the amount of PUFA was limited to 0.5 mg/ml buffered ruminal fluid, 2) the rumen fluid was not treated to remove all particles, and 3) the pH of the incubation buffer solutions remained above 6.0 over the course of incubations of LeA and LnA with rumen fluid (Fievez et al., 2007).

4.2 DISAPPEARANCE OF LEA AND LNA

Incubations with bovine ruminal fluid resulted in extensive BH of LeA (I) and LnA (III). At the end of 9 h incubations of LeA, 98.1, 97.6, 98.0, and 89.8% of 1.0, 2.5, 5.0, and 10.0 mg of added LeA had disappeared, respectively (I). At the end of the 12 h incubations of LnA, 97.4, 98.0, 98.4, and 98.5% of 2.5, 5.0, 7.5, and 10.0 mg of added LnA had disappeared, respectively (III). A remarkable amount of LeA (14.2 – 28.5%, I) and LnA (15.4 – 27.1%, III) was found to disappear at time zero, i.e., some minutes after the start of incubations with ruminal fluid, due to instantaneous formation of CLA or CLnA isomers. The proportion of LeA and LnA disappearing at time zero decreased with increases in the initial dose of LeA and LnA. In this study, the BH was stopped by placing the incubation flasks to ice-cold water. Therefore, BH proceeded some minutes in the time zero samples. In order to gain true time zero samples, the rumen fluid should be added to inactivated rumen fluid (Meynadier et al., 2018).

The rate of LeA disappearance followed the first-order exponential model at the doses from 1 to 5 mg/flask (Figure 2A, I). Increases in the LeA doses from 1 to 5 mg/flask decreased the fractional rate of BH, i.e., the rate of the fraction that can potentially disappear, from 0.55 to 0.38 1/h. However, at the highest dose (10 mg/flask), the rate of LeA BH was found to be constant at

0.08 1/h over the 9 h incubation period. The rate of LnA disappearance followed the first-order exponential model at the doses from 2.5 to 10 mg/flask (Figure 2B, III). The fractional rate LnA BH decreased from 1.99 to 0.70 1/h, respectively, over the course of 12 h incubation.

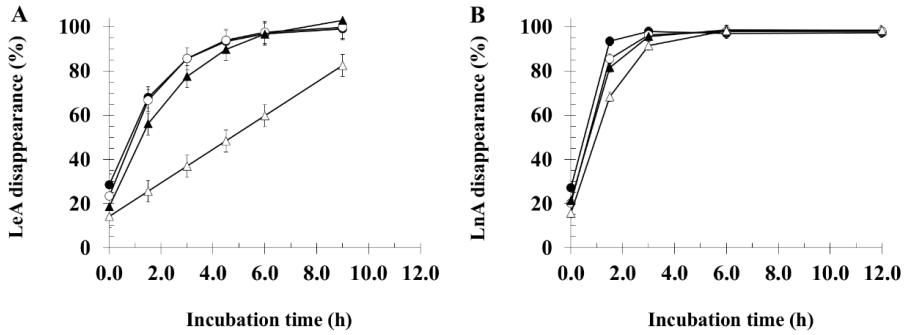

Figure 2 Temporal changes in the disappearance (% of substrate added to the incubation flask) of LeA (A, I) during incubations of 1.0 (●), 2.5 (○), 5.0 (▲), and 10.0 (Δ) mg of added LeA, and LnA (B, III) during incubations of 2.5 (●), 5.0 (○), 7.5 (▲), and 10.0 (Δ) mg of added LnA with strained rumen fluid. Values represent least squares means. SEM = 4.97 and 2.11% for LeA and LnA, respectively.

In other *in vitro* studies with rumen fluid, the fractional rates of LeA and LnA BH ranged from 0.06 to 0.48 1/h, and from 0.10 to 0.46 1/h, respectively (Table 4). The rates of LnA BH were greater than rates of LeA BH (Jouany et al., 2007). Increases in the amount of added LeA decreased the rate of LeA BH (Beam et al., 2000, Troegeler-Meynadier et al., 2003). Low pH of the incubation buffer decreased the BH rate of LeA and LnA (Troegeler-Meynadier et al., 2003). The rate of BH of LeA in soybean oil was not affected by the amount of grain or fat fed to the donor cow, or the time after feeding that ruminal inoculum was collected (Beam, et al., 2000). Extrusion of canola increased the rate of BH compared to raw canola meal (Enjalbert et al., 2003). Preconditioning (35 ◦C) linseed slowed down rate of BH compared to raw linseed (Akraim et al., 2006).

The *in vitro* conditions, initial amounts of LeA or LnA, diet of the donor cows, composition of the *in vitro* substrates, and duration of the incubation varied between different experiments. Furthermore, the rate of BH in different experiments has been calculated using a slightly different formula. Therefore, the values in different studies are comparable only within the same experiment.

Table 4. Rates of linoleic acid (LeA) and α-linolenic acid (LnA) biohydrogenation during *in vitro* incubations with rumen fluid.

Treatment	Fatty acid	Rate of BH (1/h)	Reference
Amount of added LeA	LeA	0.06-0.12	Beam, et al, 2000
	LeA	0.12-0.23	Troegeler-Meynadier et al., 2003
Raw or extruded canola	LeA	0.19-0.38	Enjalbert et al., 2003
	LnA	0.20-0.46	Enjalbert et al., 2003
Pre-conditioned, raw or extruded linseed	LeA	0.14-0.24	Akraim et al., 2006
	LnA	0.13-0.23	Akraim et al., 2006
LeA, LnA or linseed oil	LeA	0.23-0.42	Jouany et al., 2007
	LnA	0.27-0.45	Jouany et al., 2007
low pH or high pH	LeA	0.12-0.21	Troegeler-Meynadier et al., 2003
	LnA	0.10-0.23	Troegeler-Meynadier et al., 2003

4.3 BIOHYDROGENATION INTERMEDIATES FORMED FROM LEA AND LNA

4.3.1 FORMATION OF CONJUGATED 18:2 AND 18:3 ISOMERS

Cis-9,*trans*-11 18:2 and *trans*-10,*cis*-12 18:2 represented the major CLA isomers formed during incubations with LeA (Figure 3A, I). Trace amount of *cis*-9,*cis*-11 CLA, *cis*-10,*cis*-12 CLA, *trans*-9,*trans*-11 CLA, and *trans*-10,*trans*-12 CLA were also detected (I). The major CLA products formed during incubations with LnA were *trans*-11,*trans*-13 CLA, *trans*-12,*cis*-14 CLA, and *trans*-12,*trans*-14 CLA (Figure 3B, III). Minor CLA isomers to accumulate were *cis*-12,*trans*-14 CLA and *trans*-11,*cis*-13 CLA.

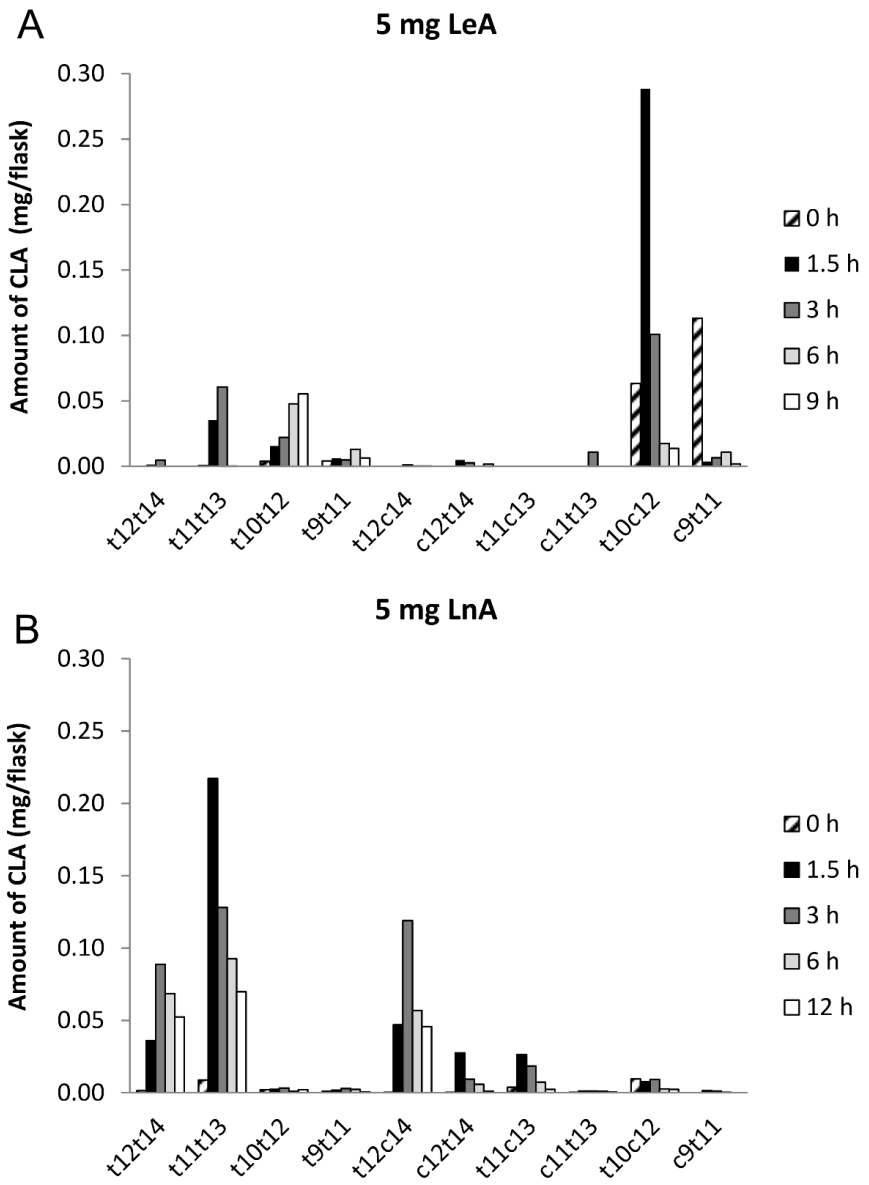

Figure 3 Temporal changes in the accumulation and disappearance of the selected conjugated linoleic acid (CLA) isomers during (A) 0 to 9 h incubations of 5.0 mg of linoleic acid (5 mg LeA, I) and (B) 0 to 12 h incubations of 5.0 mg α-linolenic acid (5 mg LnA, III) with strained bovine ruminal fluid. Values represent least squares means. The control samples without added substrate were subtracted. Pooled SEM values have been reported in publications I and III.

Incubations of LnA with rumen contents diluted with buffer prepared using H_2O or D_2O resulted in the appearance of the same CLA isomers (II). The CLA isomers accumulated and disappeared later in the presence of the D_2O (Figure 4).

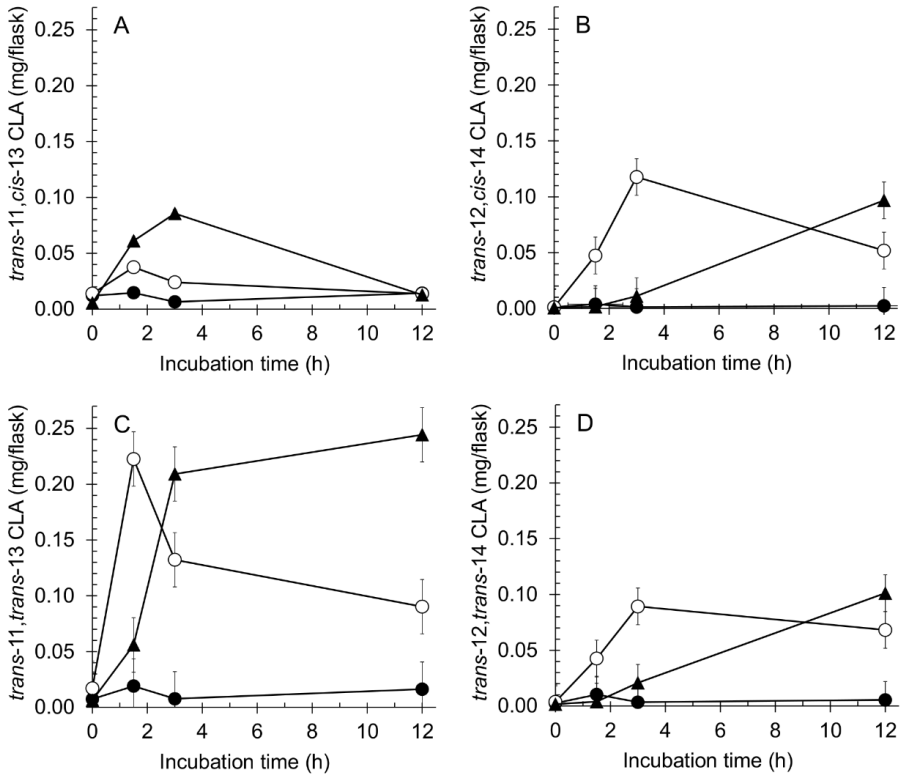

Figure 4 Formation of (A) *trans*-11,*cis*-13 CLA, (B) *trans*-12,*cis*-14 CLA, (C) *trans*-11,*trans*-13 CLA, and (D) *trans*-12,*trans*-14 CLA during 0 to 12h incubations of ground hay with strained rumen fluid diluted in de-ionised water (control) (●), rumen fluid diluted in de-ionised water and 5 mg of added α-linolenic acid (LnA) (○), or rumen fluid diluted in 56·6±1·33% moles per cent excess deuterium oxide and 5 mg of added LnA (▲) (II). Values represent least squares means. (SEM 0.003, 0.016, 0.024, and 0.008 mg/flask for *trans*-11,*cis*-13 CLA, *trans*-12,*cis*-14 CLA, *trans*-11,*trans*-13 CLA, and *trans*-12,*trans*-14 CLA, respectively).

Conjugated 18:2 isomers were found to accumulate in direct relation to the amount of added LeA (I) and LnA (III). Incremental addition of LeA resulted in a linear increase in the accumulation of *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, *cis*-9,*cis*-11 CLA, *cis*-10,*cis*-12 CLA, *trans*-9,*trans*-11 CLA, and *trans*-10,*trans*-12 CLA during incubations with bovine ruminal fluid (I). Earlier *in vitro* studies have reported increases in the amounts of the same CLA isomers during incubation of LeA with ovine rumen fluid (Jouany et al., 2007).

Incremental addition of LnA with rumen fluid increased the amount of *cis*-12,*trans*-14 CLA, *trans*-11,*cis*-13 CLA, *trans*-11,*trans*-13 CLA, *trans*-12,*cis*-14 CLA, and *trans*-12,*trans*-14 CLA in flask contents (III). Earlier *in vitro* studies have reported formation of *trans*-11,*trans*-13 CLA during incubation of LnA with ovine rumen fluid (Jouany et al., 2007) and mixed rumen microbes (Waşowska et al., 2006, Wallace et al., 2007, Or-Rashid et al., 2011)

CLnA isomers accumulated in direct relation to the dose of LnA (II, III). The main CLnA isomers to accumulate were *cis*-9,*trans*-11,*cis*-15 CLnA and *trans*-9,*trans*-11,*cis*-15 CLnA containing a conjugated diene system with one isolated double bond. The main 18:3 isomer with conjugated triene system was *trans*-9,*trans*-11,*cis*-13 CLnA (II). Increases in the dose of LnA increased the amount of *cis*-9,*trans*-11,*cis*-15 CLnA, *trans*-9,*trans*-11,*cis*-15 18:3, and *trans*-9,*trans*-11,*cis*-13 18:3 (III). In literature, increases in camelina oil in diet increased the amount of *cis*-9,*trans*-11,*cis*-15 CLnA, and three other geometric Δ9,11,15 CLnA isomers in bovine milk fat (Halmemies-Beauchet-Filleau et al., 2017). *Cis*-9,*trans*-11,*cis*-15 18:3 has been detected in milk fat from ewes (Gómez-Cortés et al., 2009) and cows (Lerch et al., 2012a) fed linseeds, a rich source of LnA.

A trace amount of Δ10,12,15 CLnA isomer, a missing intermediate of *trans*-10 shifted rumen LnA BH pathway, was detected in flask contents after incubations with LnA (II, III). The 12 amu gaps at ion fragments *m*/*z* 210 and 222, *m*/*z* 236 and 248, *m*/*z* 276 and 288, and the M^+ at *m*/*z* 331 in the mass spectrum of the DMOX derivatives located the double bonds at Δ10, 12, and 15 with 18:3 structure, respectively (II). Flask content of Δ10,12,15 CLnA increased in flasks incubated for 1.5 h, and decreased thereafter (II, III). The increases in the mean amount of Δ10,12,15 CLnA were detected only between 2.5 and 5.0 mg of added LnA. Increasing the dose of LnA from 5.0 mg to 10 mg did not increase the amount of the Δ10,12,15 CLnA. Furthermore, the differences in the amounts of Δ10,12,15 CLnA were detected only in the samples incubated for 1.5 h. It is possible that the greatest accumulation of Δ10,12,15 CLnA is before or after the detected 1.5 h incubation time point. However, the amount of Δ10,12,15 CLnA was very low and did not clearly increase with increasing amount of added LnA. Therefore, it is not possible to confirm, whether the Δ10,12,15 CLnA isomer is a product of LnA BH.

4.3.2 FORMATION OF NON-CONJUGATED 18:2 AND 18:3 ISOMERS

4.3.2.1 Identification of NC 18:2 and 18:3 isomers

During GC analysis of FAME several unknown fatty acid isomers were found to elute just before LeA (I) and LnA (II, III). GC-MS analysis of the FAME of these fatty acid isomers revealed a molecular ion at *m/z* 294 or 292 confirming an 18-carbon fatty acid structure containing two or three double bonds, respectively. The electron impact ionization mass spectrum of all DMOX derivatives contained intense peaks at *m/z* 113 and 126, typical of oxazoline derivatives. Molecular ions at *m/z* 333 and 331 provided unequivocal evidence that the unknown products formed during incubations of LeA and LnA with rumen fluid were isomers of 18:2 and 18:3, respectively.

The main novel fatty acid isomers formed during incubations with LeA in rumen fluid were identified as *cis*-7,*cis*-12 18:2 and *cis*-8,*cis*-12 18:2 (I). The main novel products formed during incubations with LnA were identified as *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3 (II). The mass spectra of DMOX derivatives of the NC *cis*-7,*cis*-12 18:2, *cis*-8,*cis*-12 18:2, and *cis*-8,*cis*-12,*cis*-15 18:3 are shown in Figures 5, 6, and 7, respectively. For example, a fatty acid with a prominent ion fragment of 100% abundance at *m/z* 222 in the mass spectrum (Figure 6) indicates a bis-methylene interrupted Δ8,12 double bond system representing a cleavage between carbon atoms 9 and 10, i.e., the centre of the bis-methylene interrupted bond system. Double-bond positions of Δ8 and Δ12 for isomers of Δ8,12 18:2 were indicated based on 12-amu intervals between *m/z* 182 and 194, and *m/z* 236 and 248, respectively. Characteristic ion fragments of other fatty acid isomers recorded during GC-MS analysis of DMOX derivatives of fatty acids formed during incubations of LeA and LnA with bovine rumen fluid are reported in I and in Table 5, respectively.

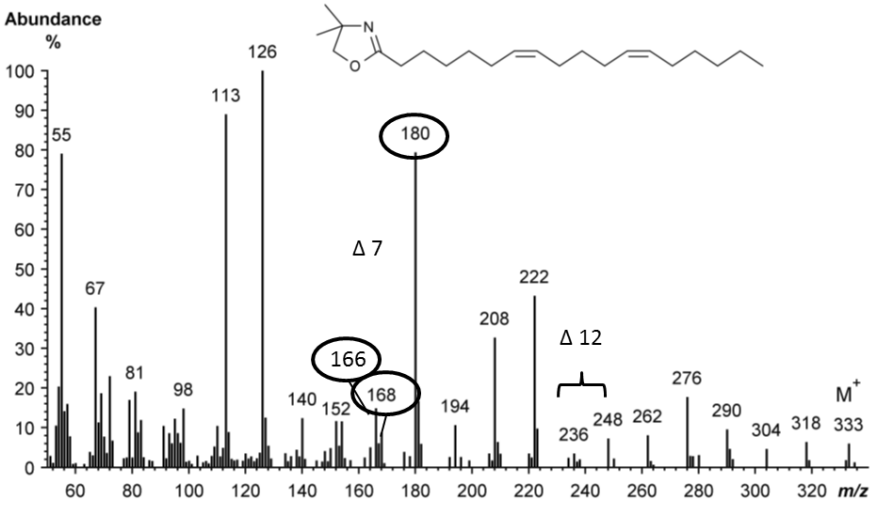

Figure 5 Mass spectrum of the 4,4-dimethyloxazoline derivative of *cis*-7,*cis*-12 18:2 formed during incubations of linoleic acid with strained bovine rumen fluid (I). A molecular ion at *m*/*z* 333 confirmed the octadecadienoic acid structure. Gaps of 12 atomic mass units between *m*/*z* 168 and 180, and *m*/*z* 236 and 248 located double bonds at Δ7 and 12, respectively.

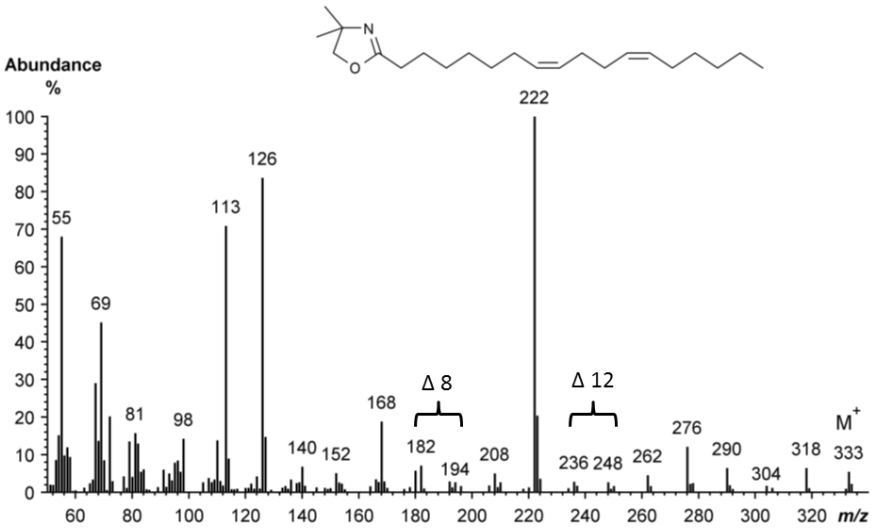

Figure 6 Mass spectrum of the 4,4-dimethyloxazoline derivative of *cis*-8,*cis*-12 18:2 formed during incubations of linoleic acid with strained bovine rumen fluid (I). A molecular ion at *m*/*z* 333 confirmed an octadecadienoic acid structure. Gaps of 12 atomic mass units between *m*/*z* 182 and 194, *m*/*z* 236 and 248, and an abundant ion at *m*/*z* 222 confirmed a bis-methylene interrupted Δ8,12 double bond arrangement.

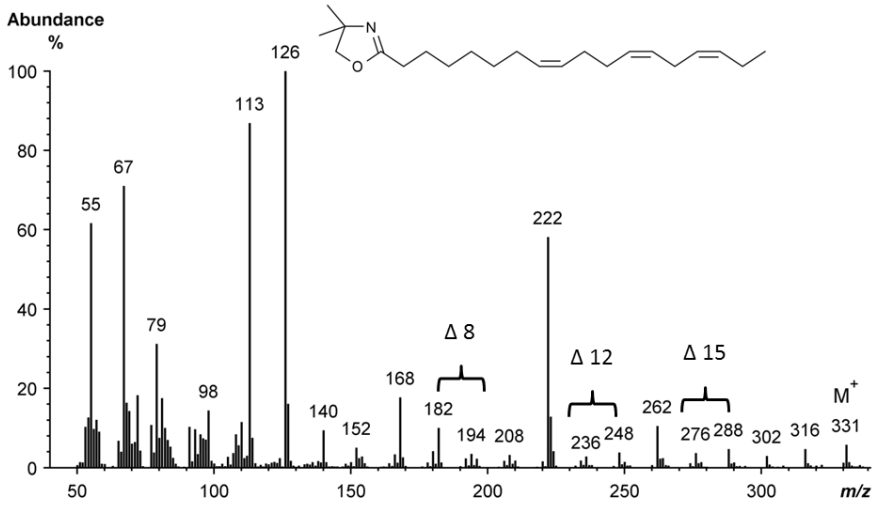

Figure 7 Mass spectrum of the 4,4-dimethyloxazoline derivative of *cis*-8,*cis*-12,*cis*-15 18:3 formed during incubations of α-linolenic acid with strained bovine rumen fluid (II). A molecular ion at *m*/*z* 331 confirmed an octadecatrienoic acid structure. Gaps of 12 atomic mass units between *m*/*z* 182 and 194, *m*/*z* 236 and 248, and an abundant ion at *m*/*z* 222 confirmed a Δ8,12 double bond arrangement. The double bond at Δ15 was located based on a gap of 12 atomic mass units at *m*/*z* 276 and 288.

Table 5. Characteristic ion fragments recorded during gas chromatography-mass spectrometry analysis of DMOX derivatives of fatty acids formed during incubations of α-linolenic acid with strained bovine rumen fluid (III).

Fatty acid	Characteristic ion fragments, *m/z* (relative intensity)
cis-12,*cis*-15 18:2	113 (100), 126 (99), 238(3), 250 (3), 278 (16), 290 (7), 333 (25, M+)
trans-12,*cis*-15 18:2	113 (100), 126 (94), 238(6), 250 (3), 278 (36), 290 (5), 333 (23, M+)
Δ4,12,15 18:3	113 (100) ,126 (73), 139 (3), 166 (22), 180 (41) 236 (2), 248 (5), 276(10), 288 (9), 331 (5, M+)
Δ5,12,15 18:3	113 (100) ,126 (57), 153 (6), 236 (3), 248 (2), 276(5), 288 (2), 331 (5, M+)
Δ9,11,15 18:3a	113 (52), 126 (51), 196 (4), 208 (2), 222 (6), 234 (1), 262 (100), 276 (2), 288 (1), 331 (32, M^+)
Δ9,11,15 18:3b	113 (82), 126 (56), 196 (4), 208 (2), 222 (4), 234 (2), 262 (100), 276 (2), 288 (1), 331 (24, M^+)
Δ10,12,15 18:3	113 (100), 126 (58), 210 (4), 222 (2), 236 (4), 248 (2), 276 (1), 288 (1), 331 (16, M^+)
cis-7,*cis*-12,*cis*-15 18:3	113 (79), 126 (100), 168 (7), 180 (40), 236 (2), 248 (4), 276 (7), 288 (6), 331 (4, M+)
cis-8,*cis*-12,*cis*-15 18:3	113 (87), 126 (100), 182 (13), 194 (5), 222 (91), 236 (5), 248 (7), 276 (8), 288 (10), 331 (17, M+)
cis-9,*trans*-11,*cis*-15 18:3	113 (76), 126 (99), 196 (4), 208 (3), 222 (4), 234 (3), 262 (100), 276 (1), 288 (1), 331 (25, M^+)
trans-8,*cis*-12,*cis*-15 18:3	113 (100), 126 (74), 182 (10), 194 (3), 222 (67), 236 (5), 248 (4), 276 (11), 288 (5), 331 (13, M+)
trans-9,*trans*-11,*cis*-15 18:3	113 (23), 126 (24), 196 (1), 208 (1), 222 (1), 234 (1), 262 (100), 276 (1), 288 (1), 331 (20, M^+)

DMOX, 4,4-dimethyloxazoline

4.3.2.2 Accumulation of NC 18:2 and 18:3

Several NC 18:2 and 18:3 isomers were found to accumulate in direct relation to the amount of added LeA (I) or LnA (III). The main novel NC 18:2 isomers to accumulate during incubations with LeA were *cis*-7,*cis*-12 18:2 and *cis*-8,*cis*-12 18:2. The main novel NC 18:3 isomers to accumulate during incubations with LnA were *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3. Temporal changes in these BH products formed during incubation of incremental doses of LeA or LnA with rumen fluid are shown in Figure 8.

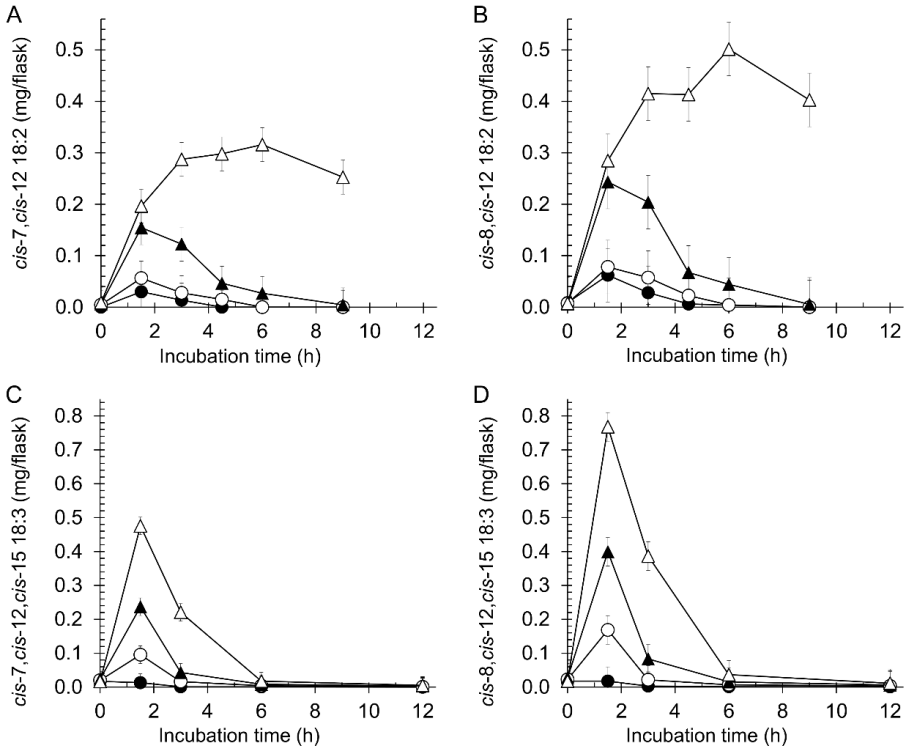

Figure 8 Temporal changes in the accumulation of (A) *cis*-7,*cis*-12 18:2, and (B) *cis*-8,*cis*-12 18:2 during incubations of 1.0 (●), 2.5 (○), 5.0 (▲), and 10.0 (Δ) mg of LeA (I), and (C) *cis*-7,*cis*-12,*cis*-15 18:3, and (D) *cis*-8,*cis*-12,*cis*-15 18:3 during incubations of 2.5 (●), 5 (○), 7.5 (▲), and 10.0 (Δ) mg of LnA (III) with strained bovine ruminal fluid. (SEM 0.033, 0.052, 0.026, and 0.042 mg/flask for *cis*-7,*cis*-12 18:2, *cis*-8,*cis*-12 18:2, *cis*-7,*cis*-12,*cis*-15 18:3, and *cis*-8,*cis*-12,*cis*-15 18:3, respectively).

Incremental doses of LeA from 1 to 10 mg/flask resulted in quadratic increases in the accumulation of *cis*-7,*cis*-12 18:2 and *cis*-8,*cis*-12 18:2, and linear increases in *cis*-6,*cis*-12 18:2, *trans*-8,*cis*-12 18:2 + Δ4,12 18:2, and *trans*-9,*cis*-12 18:2 during incubations with bovine ruminal fluid. Incremental

doses of LnA resulted in quadratic increases in the accumulation of *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3. *Cis*-12,*cis*-15 18:2 and *trans*-11,*cis*-15 18:2 were the main NC 18:2 products formed during incubations of LnA with smaller amounts of *trans*-10,*cis*-15 18:2, *trans*-11,*trans*-15 18:2, and *trans*-12,*cis*-15 18:2. Incremental doses of LnA resulted in quadratic increases in the accumulation of *trans*-11,*cis*-15 18:2, and linear increases in the accumulation of *cis*-12,*cis*-15 18:2, *trans*-10,*cis*-15 18:2, *trans*-11,*trans*-15 18:2, and *trans*-12,*cis*-15 18:2.

Cis-12,*cis*-15 18:2 isomer has been identified from linseed oil-fed lamb meat (Alves and Bessa, 2007), muscle of beef cows (Nassu et al., 2011) and milk fat of goats (Gómez-Cortés et al., 2018). Supplements of extruded linseed oil (Rego et al., 2009) or linseeds (Lerch et al., 2012b) rich in LnA increased milk *cis*-12,*cis*-15 18:2 content. *Cis*-12,*cis*-15 18:2 has been proposed to be a BH intermediate product of LnA formed from direct reduction of *cis*-9 double bond of LnA (Bessa et al., 2007). In the current experiment (III), the amount of the *cis*-12,*cis*-15 18:2, *trans*-10,*cis*-15 18:2, and *trans*-11,*cis*-15 18:2 increased in direct relation to the amount of added LnA in bovine rumen fluid, which confirms that *cis*-12,*cis*-15 18:2 is an intermediate of the LnA BH in rumen and explains the origin of these fatty acid isomers in ruminant derived foods.

The percentages of the *trans*-10,*cis*-15 18:2 of the total amount of *trans*-10,*cis*-15 18:2 and *trans*-11,*cis*-15 18:2 in the samples after 1.5 h incubation with 2.5, 5.0, and 7.5 mg of added LnA and after 3 h incubation with 10 mg of added LnA were 37.6, 50.5, 47.8, and 44.3%, for 2.5, 5.0, 7.5, and 10.0 mg of added LnA, respectively (SEM = 2.07%). The percentage increased from 2.5 to 5.0 mg of added LnA but remained the same when the doses of added LnA increased from 5.0 to 10 mg. No *trans*-10,*cis*-15 18:2 was detected in flask contents for control samples or any samples at the beginning of the incubations.

4.3.3 FORMATION OF 18:1 ISOMERS AND STEARIC ACID

Incremental addition of LeA resulted in the linear accumulation of several 18:1 intermediates in incubation flasks, including *cis*-12 18:1, *trans*-10 18:1, and *trans*-11 18:1 (Figure 9A), consistent with previous observations (Harfoot and Hazlewood, 1997, Enjalbert et al., 2003, Jouany et al., 2007). All 18:1 isomers from *trans*-4 18:1 to *trans*-16 18:1, and from *cis*-9 18:1 to *cis*-16 18:1 accumulated during the incubations of LnA (III). The main 18:1 isomer was unresolved *trans*-13/14 18:1 (Figure 9B) consistent with previous observations (Jouany et al., 2007).

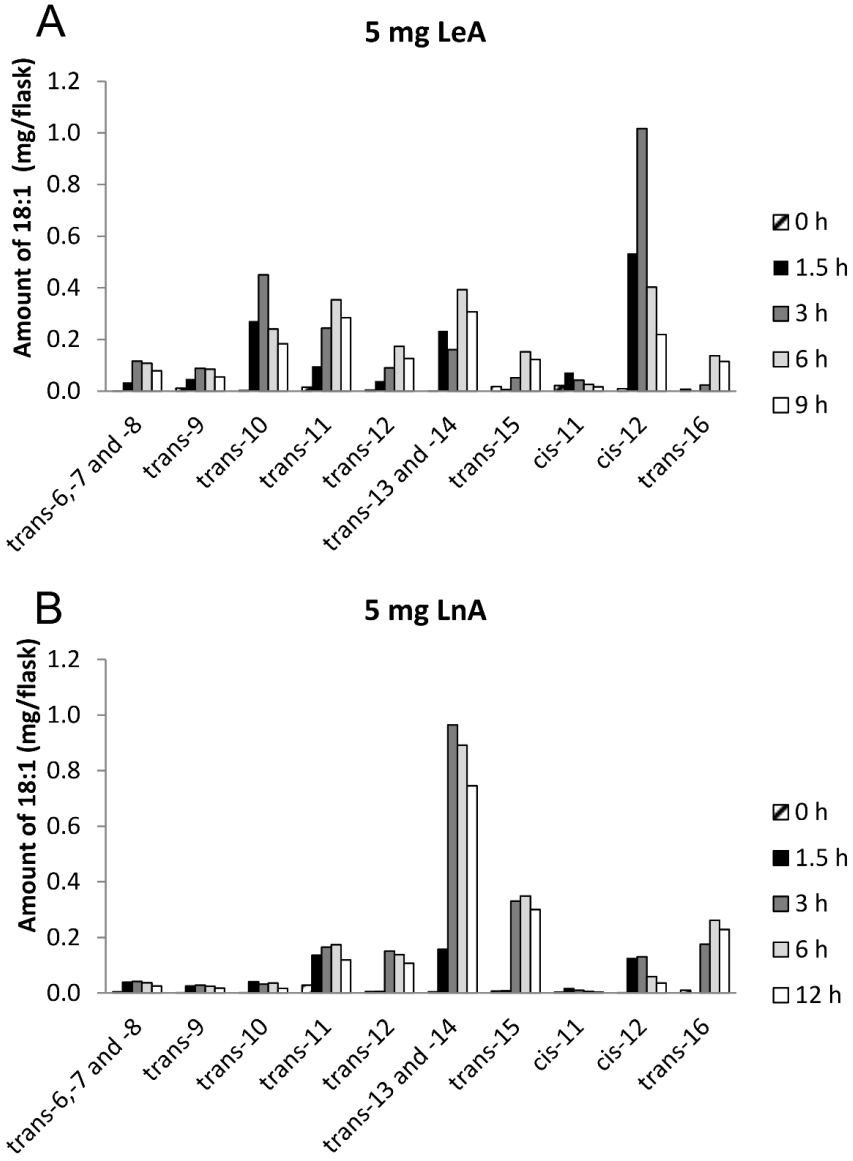

Figure 9 Temporal changes in the accumulation and disappearance of the selected 18:1 intermediate products during (A) 0 to 9 h of 5.0 mg of linoleic acid (5 mg LeA, I) and (B) 0 to 12 h incubations α-linolenic acid (5 mg LnA, III) with strained bovine ruminal fluid. Values represent least squares means. Pooled SEM values have been reported in publications I and III.

The appearance of *cis*-12 18:1 formed during incubations of LeA with ruminal fluid could be considered as evidence of BH involving the direct reduction of *cis*-9 double bond, but it is also possible that *cis*-12 18:1 originates from the BH of *trans*-10,*cis*-12 CLA (McKain et al, 2010) or the NC 18:2 intermediates. Sunflower oil supplementation, a rich source of LeA in diet, increased the concentrations of *cis*-12 18:1 in bovine omasal digesta (Shingfield et al., 2008b) and in lamb meat (Bessa et al., 2007).

Increasing the amounts of added LeA resulted in a linear increase in the mean ratio of *trans*-10 18:1:*trans*-11 18:1 in incubation flasks over the 9-h incubation period (0.787, 0.889, 1.076, and 1.671, SEM = 0.315, for 1.0, 2.5, 5.0, and 10.0 mg of added LeA, respectively). Under the controlled conditions of this experiment, the shift from *trans*-11 to *trans*-10 pathway during BH of LeA occurred over a relatively short incubation period. Previous reports have indicated that decreases in pH promote the formation of *trans*-10,*cis*-12 CLA and *trans*-10 18:1 from LeA by ruminal microbes *in vitro* (Troegeler-Meynadier et al., 2003, Fuentes et al., 2009). In the previous reports, the mean ratio of *trans*-10 18:1:*trans*-11 18:1 was between 0.019 and 0.036 (Troegeler-Meynadier et al., 2003) and between 0.19 and 34.2 (Fuentes et al., 2009). Isomerization of LeA to CLA was strongly inhibited by a low ruminal pH (pH under 6.0 vs. pH 6.5) and by increasing concentrations of LnA in the incubation media (Troegeler-Meynadier et al., 2003). However, in this study (I, III), the mean decrease in pH over the course of incubations was marginal (0.4 unit from pH 6.6 to 6.2) and independent of the amount of LeA or LnA added. In another *in vitro* study, the the mean ratio of *trans*-10 18:1:*trans*-11 18:1 was low (0.12) when no LeA was added to incubation cultures (Zened et al., 2012). A large accumulation of *trans*-10 fatty acids was only observed with an adapted microflora, as well as with an addition of non-esterified LeA to the incubation substrate (the mean ratio of *trans*-10 18:1:*trans*-11 18:1 13.2) (Zened et al., 2012). In this study (I), the relatively moderate *trans*-10 shift was observed with increasing the amount of LeA to the incubation substrate without microflora adaptation.

Trans-11 18:1, Δ12 18:1, *trans*-13/14 18:1, Δ15 18:1, and Δ16 18:1 isomers accumulated with increasing dose of LnA (III). Supplements of extruded linseed oil (Rego et al., 2009) or linseeds (Collomb et al., 2004, Lerch et al., 2012b, Kliem etl al., 2017) rich in LnA increased milk *cis*-12 18:1, *cis*-15 18:1, *trans*-11 18:1, *trans*-12 18:1, *trans*-13/14 18:1, *trans*-15 18:1, *trans*-16 18:1 content *in vivo*.

Some of the 18:1 isomers were further reduced to 18:0, which is the end product of the BH of LeA (I) and LnA (II). Complete reduction of LeA to 18:0 was inhibited in direct relation to the amount of added substrate or intermediate product formed from LeA. The extent of inhibition was greatest for the highest amount of LeA addition (I). The 18:0 was detected as end product of LnA BH (II). However, incremental dose of LnA did not increase the accumulation of 18:0 in this experiment (III).

The amount of *cis*-9 18:1 was also found to increase with increasing dose of LeA (I) and LnA (III). The amount of *cis*-9 18:1 was high at the beginning of the incubations declining thereafter. In all three experiments, the LeA (I) or LnA (II, III) was added to the incubation flasks as a suspension in aqueous Tween 80 and NaOH. However, Tween 80 (polyoxyethylene sorbitan monooleate) is a source of *cis*-9 18:1 (Ilko et al., 2015). The dose of the Tween 80 increased in the same ratio with the amount of added LeA or LnA. The control samples without added LnA contained the same amount of Tween 80 as the samples with 5.0 mg of added LnA. Therefore, increases in the amount of *cis*-9 18:1 with increasing dose of LeA or LnA can be explained by the increases in the amount of Tween 80. BH of *cis*-9 18:1 by ruminal microbes involves the formation of several positional *trans* 18:1 isomers and 18:0 (Mosley et al., 2002) or 10-O 18:0 (McKain et al, 2010). Therefore, the 18:1 isomers, 18:0, and 10-O 18:0 accumulated in these studies (I-III) must be interpreted with caution.

4.4 ENRICHMENT OF LNA BIOHYDROGENATION PRODUCTS AND BIOHYDROGENATION MECHANISMS

Isomerization of LeA to *cis*-9,*trans*-11 CLA or *trans*-10,*cis*-12 CLA is known to be the first step of BH of LeA (Wallace et al., 2007). Clear differences have been identified between the mechanisms underlying the synthesis of *cis*-9,*trans*-11 18:2 and *trans*-10,*cis*-12 18:2 formed from LeA (Wallace et al., 2007). In this study, the mechanism of the BH of LnA was studied by incubating LnA with a buffer prepared using D_2O instead of de-ionized H_2O (II). Mass spectra of the intermediate products formed during the incubations were analysed to investigate possible interactions with D_2O and to detect the number and location of ^2H labels in the fatty acid moiety. Enrichment of ^2H (n+1, n+2 or n+3) indicating the number of ^2H labels in the selected BH products formed is shown in Figure 10. A summary of the major BH pathway of LnA in this fermentation environment indicating locations of the ^2H labels in the carbon chains of the BH products is shown in Figure 11.

The mass spectrum of the DMOX derivative of *cis*-9,*trans*-11,*cis*-15 CLnA and *trans*-9,*trans*-11,*cis*-15 CLnA formed during incubations of LnA with buffer prepared using D_2O located the incorporation of one ^2H (n+1) on C-13 of the FA moiety (II). *Cis*-9,*trans*-11,*cis*-15 18:3 and other geometric isomers of Δ9,11,15 18:3 were formed from LnA via a mechanism that appears identical to that responsible for the conversation of LeA to geometric isomers of Δ9,11 CLA, indicating a ^2H label on C-13 (Wallace et al., 2007). The appearance of a single label on C-13 of 9,11 CLA isomers formed from LeA was explained by H abstraction on C-11, which for reasons of thermodynamic stability was followed by re-arrangement of the double bond and the assimilation of a

proton from water (Kepler and Tove, 1967, Wallace et al., 2007, McIntosh et al., 2009). A common mechanism for the synthesis of Δ9,11 CLA from LeA and Δ9,11,15 CLnA from LnA during incubations with mixed rumen microbiota is not unexpected, given that the same Δ^{12}-*cis*,Δ^{11}-*trans* isomerase isolated from *B. fibrisolvens* is capable of both reactions (Kepler et al., 1971).

The molecular ion of the DMOX derivative of *trans*-11,*cis*-15 18:2 formed during incubations of LnA with D_2O at *m/z* 335 (n+2) confirmed the incorporation of two ^2H labels in the FA moiety (II). Relative abundances of ion fragments at *m/z* 210 and 211, and at *m/z* 264, 265 and 266 located the incorporation of a ^2H label on C-9 and another on C-13 (Figure 11).

Relative abundance of n+1 isotopomers of Δ10,12,15 CLnA was higher compared with natural enrichment, but the position of the ^2H label could not be located (II). Furthermore, concentrations of Δ10,12,15 CLnA were too low to allow the MPE at n+2 to be estimated accurately. However, an increase in the n+2 isotopomer indicates that transformation of LnA to *trans*-10,*cis*-15-18:2 by rumen microbiota involves exchange of two H ions from water.

Incubations of LnA with rumen contents resulted in the formation of *trans*-9,*trans*-11,*cis*-13 CLnA (II, III). Enrichment of the n+1 isotopomer of *trans*-9,*trans*-11,*cis*-13 CLnA increased over the course of 12 h incubations, but was consistently lower compared with incorporation of ^2H in Δ9,11,15 CLnA products (Figure 10, II). Nevertheless, an increase in the abundance of the n+1 isotopomer above natural enrichment suggests that the conversion of *trans*-9,*trans*-11,*cis*-13 CLnA from LnA involves an exchange of H with water, by a mechanism that apparently differs from Δ9,11,15 CLnA formation (II).

In this study, accumulation of NC 18:2 and 18:3 isomers provided the first indications that hydrogenation of LeA and LnA by ruminal bacteria may also proceed via migration of *cis*-9 double bond rather than isomerization of the *cis*-12 double bond by the Δ^{12}-cis,Δ^{11}-trans isomerase enzyme. The mass spectra of the DMOX derivatives of *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3 formed during incubations of LnA with D_2O provided no evidence of ^2H labelling (II).

The mass spectrum of the DMOX derivative of *cis*-12,*cis*-15 18:2 (II) formed in the presence of D_2O indicated a molecular ion at *m/z* 336 (n+3), confirming an octadecadienoic acid structure and incorporation of three ^2H labels (Figure 10). The location of each label could not be determined accurately. One of the ^2H labels was located on *m/z* 127 (126 +1). However, the *m/z* 126 is a characteristic ion fragment of DMOX derivatives which is thought to be formed due to a cyclization-displacement reaction and cleavage between carbon atoms 4 and 5 (Spitzer, 1996, Christie, 1998). The mass spectrum of the DMOX derivative of *trans*-11,*trans*-13 CLA indicated the incorporation of two or three ^2H labels (Figure 10) located between C-9 and C-16 (II). The mass spectrum of the DMOX derivative of *trans*-12,*trans*-14 CLA revealed a molecular ion at *m/z* 336 (n+3), indicating incorporation of three ^2H atoms, but the locations could not be established (II).

Due to the extensive ^{2}H labelling of 18:1 intermediates during incubations with D_2O, it was not possible to separate individual 18:1 isomers during GC analysis to quantify the amounts (mg/flask) and ^{2}H enrichments (II). The peaks of the several times ^{2}H labelled 18:0 and 18:1 isomers in GC-FID and GC-MS chromatograms were wider than normal fatty acid peaks. The peaks had shoulders on left side. The more ^{2}H the fatty acid contained the wider the shoulder was. The ^{2}H labelled fatty acid eluted from GC before the normal fatty acid, due to the ^{2}H labelled material being on the left side of the chromatogram peak as a shoulder and the unlabelled fatty acid being on the right side of the peak. This made the quantification of the several times ^{2}H labelled peaks difficult. However, the mass spectrum of the FAME derivative of *trans*-11 18:1 indicated one ^{2}H from *m/z* 211 (210+1) onwards, two ^{2}H from *m/z* 266 (264+2) onwards, three ^{2}H from *m/z* 295 (292+3) onwards, indicating ^{2}H labels on C-9, C13, and C-15 (Figure 11), confirming that one of the sources of this isomer is the hydrogenation of the *cis*-15 double bond of the *trans*-11,*cis*-15 18:2, which has ^{2}H labels on C-9 and C-13.

Besides *trans*-11 18:1, the other 18:1 isomers to be formed were *cis*-15 18:1, *trans*-12 18:1, *trans*-13/14 18:1, *trans*-15 18:1, and *trans*-16 18:1 (III). The exact source of these isomers remains unknown. Based on putative BH pathways of LnA, Bessa et al. (2007) expected the levels of *trans*-14 18:1 to increase with increases in *cis*-12,*cis*-15 18:2. In this *in vitro* study, the amount of *cis*-12,*cis*-15 18:2 increased with increases in initial dose of LnA. Furthermore, the mass spectrum of the *trans*-13/14 18:1 and *cis*-15 18:1 DMOX derivatives formed during incubations of LnA with D_2O indicated that one of the ^{2}H labels was located on *m/z* 127 (126 +1). Therefore, one of the origins of *trans*-13/14 18:1 and *cis*-15 18:1 could be *cis*-12,*cis*-15 18:2, which had also ^{2}H label on the *m/z* 127 (126 +1) (II).

The mass spectra of the DMOX derivative of the 18:0 at *m/z* 342 (337+5) indicated five 2H labels after incubation of LnA with rumen fluid and buffer prepared using D2O (II). The mean labelling of the 18:0 FAME at n+1, n+2, n+3, n+4, and n+5 were 0.21, 0.27, 0.24, 0.18, and 0.12 (SEM 0.012, 0.012, 0.007, 0.004, and 0.002, respectively).

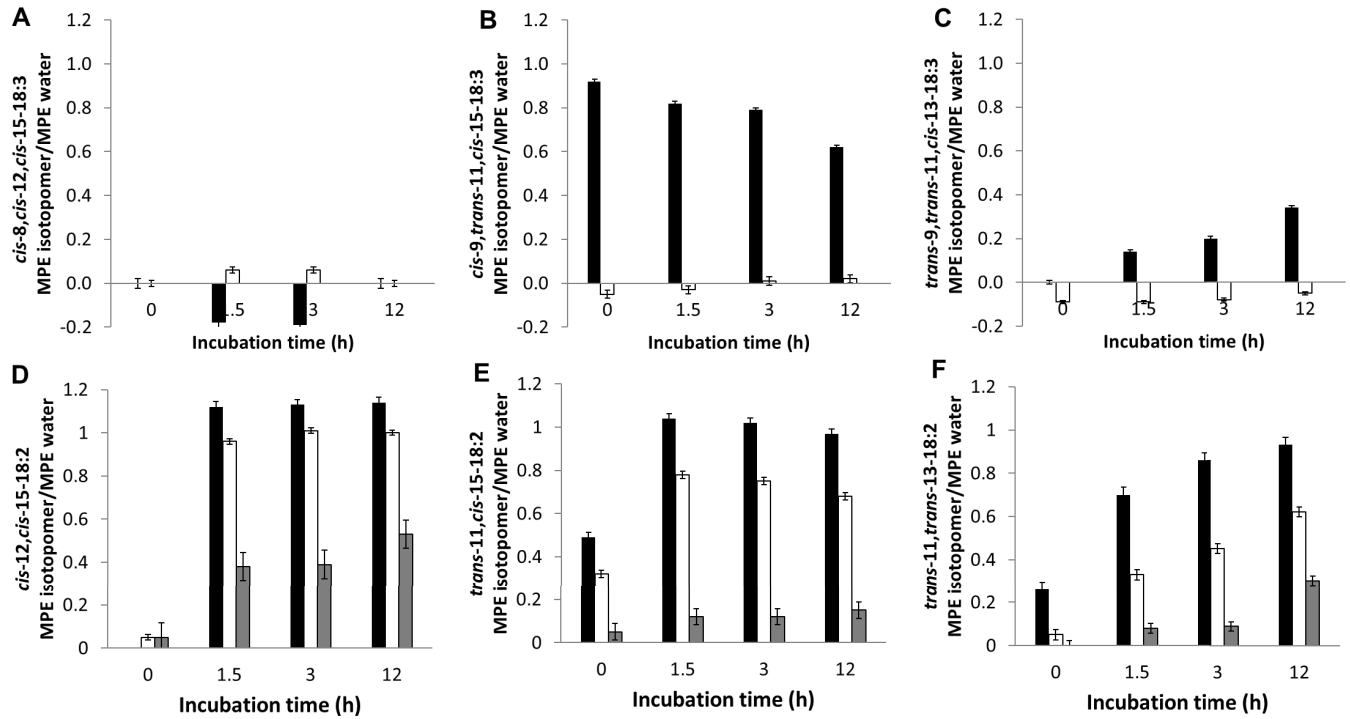

Figure 10 Enrichment in n+1 (black), n+2 (white), and n+3 (grey) isotopomers of (A) *cis*-8,*cis*-12,*cis*-15 18:3, (B) *cis*-9,*trans*-11,*cis*-15 18:3, (C) *trans*-9,*trans*-11,*cis*-15 18:3, (D) *cis*-12,*cis*-15 18:2, (E) *trans*-11,*cis*-15 18:2, and (F) *trans*-11,*trans*-13 CLA synthesized from LnA during incubations with bovine ruminal fluid (II). Enrichment calculated from the ratio of moles per cent excess (MPE) in the incubation product / MPE in water. Mean enrichment in water was 56,6% ± 1,33% MPE. Values are least square means and SEM for 12 observations.

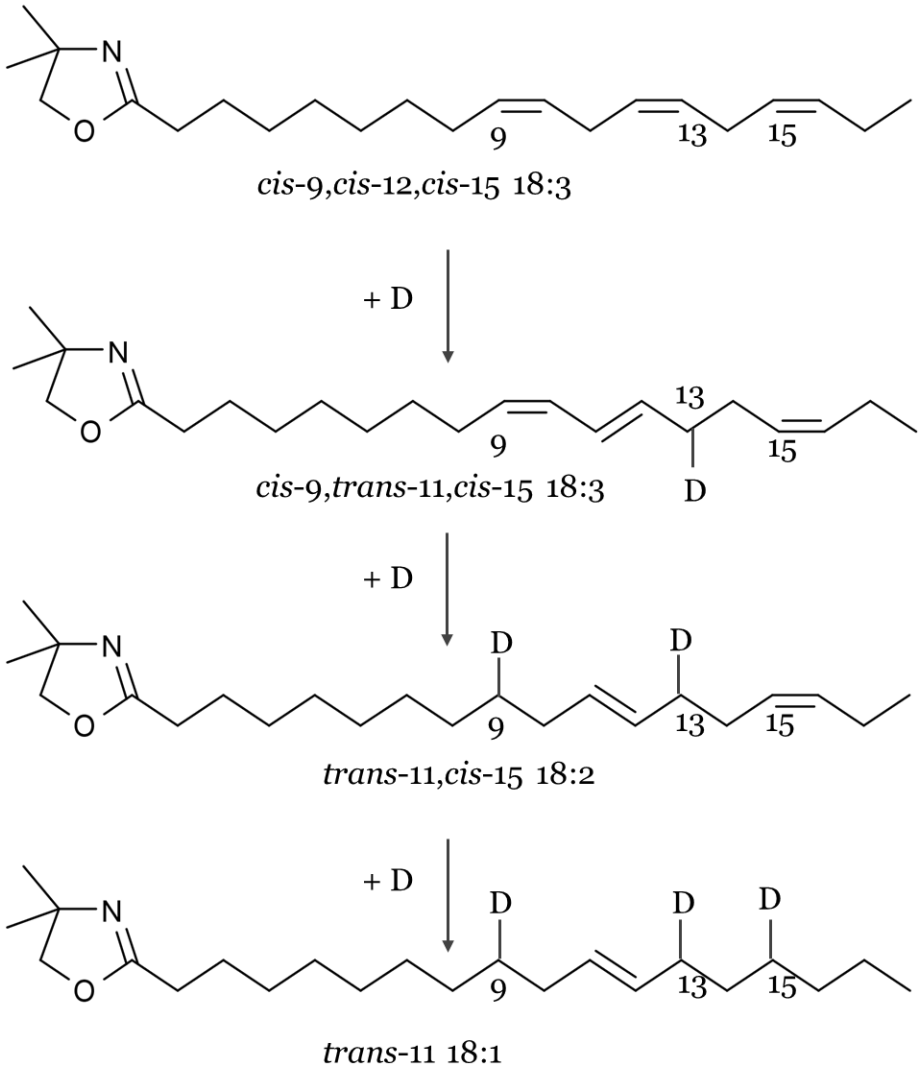

Figure 11 Biohydrogenation of α-linolenic acid (*cis*-9,*cis*-12,*cis*-15 18:3) during incubations with D_2O containing buffer (II). The 'D' stands for the deuterium label (^2H) in carbon chain of the DMOX derivative of the fatty acid isomers. The *cis*-9,*trans*-11,*cis*-15 18:3 located the incorporation of ^2H on C-13 of the FA moiety. *Trans*-11,*cis*-15 18:2 located the incorporation of a single ^2H on C-9 and another on C-13. Mass spectrum of the *trans*-11 18:1 indicated three ^2H labels located on C-9, C-13, and C-15.

4.5 FRACTIONAL RATES OF TRANSFER AMONG THE FATTY ACID POOLS

Estimation of transfer rates among fatty acid pools based on the appearance of BH intermediates during incubations of 5 and 10 mg of LeA with bovine ruminal fluid indicated that the transfer rate of LeA to the CLA pool was almost identical to the rate of LeA transfer to the NC 18:2 isomer pool (Figure 12). However, the CLA pool was, in the main, comprised of *trans*-10,*cis*-12 CLA, whereas the conversion of LeA to *cis*-9,*trans*-11 CLA was found to occur almost instantaneously. It would, therefore, appear that the kinetics of LeA isomerization to Δ10,12 geometric isomers of CLA are similar to the rates of LeA conversion to NC 18:2. The rate of CLA isomer reduction in this experiment was lower than values observed in another *in vitro* study, when the production of CLA did not account for LeA being converted to NC 18:2 intermediates (Ribeiro et al., 2007). The rates of transfer from the CLA to 18:1 pool were higher compared with the corresponding rates of transfer from the NC 18:2 to 18:1 pool. Furthermore, increases in LeA addition had much smaller inhibitory effects on the rate of transfer from the CLA to 18:1 pool compared with the rate of transfer from the NC 18:2 to 18:1 pool.

The transfer rates from the 18:3 pool to the 18:2 pool during incubations with LnA were higher than the rates of the 18:2 transfer to the 18:1 isomer pool (Figure 12). LnA, CLnA, and NC 18:3 were assigned to one 18:3 pool because LnA was rapidly converted to CLnA isomers and the transfer rate from LnA to *cis*-9,*trans*-11,*cis*-15 CLnA, the main CLnA isomer, could not be detected.

The transfer rates from the 18:1 to 18:0 pool were estimated to be lower than other steps of LeA or LnA BH by bovine ruminal fluid and decreased by increases in LeA or LnA addition, in agreement with earlier reports (Troegeler-Meynadier et al., 2003). The addition of 10 mg of LeA inhibited the transfer rate from the 18:1 pool more than the addition of LnA. However, the distribution of the different kind of *cis* and *trans* 18:1 isomers is different with incubations with LeA and LnA. All transfer rates decreased with increases in LeA or LnA addition.

Again, as the LeA or LnA was added to the incubation flasks as a suspension in aqueous Tween 80, a source of *cis*-9 18:1, and the BH of *cis*-9 18:1 by ruminal microbes involves the formation of several positional *trans* 18:1 isomers and 18:0, the fractional rates calculated in these studies (I, III) must be interpreted with caution. Furthermore, due to different basal substate creating different *in vitro* conditions, the values in different studies are comparable only within the same experiment.

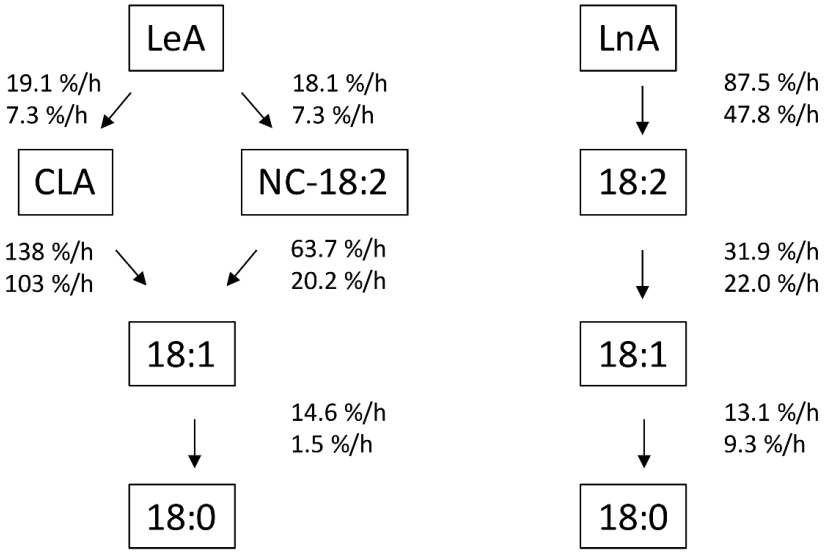

Figure 12 Estimates of the transfer rates calculated by a dynamic multi-compartmental model WinSAAM among fatty acid pools with 5 mg (the upper value) or 10 mg (the lower value) of added linoleic acid (I) or alpha-linolenic acid (III) during incubations with bovine ruminal fluid. Boxes represent fatty acid pools and arrows indicate the transfer of fatty acids between pools during biohydrogenation: linoleic acid (LeA), alpha-linolenic acid including other 18:3 isomers (LnA), geometric isomers of conjugated linoleic acid (CLA), non-conjugated 18:2 isomers (NC 18:2), all 18:2 isomers (18:2), *cis* and *trans* 18:1 isomers (18:1), and stearic acid (18:0).

5 CONCLUSIONS

The present doctoral thesis work investigated the structure of the major and minor fatty acid isomers formed from LeA and LnA, some of which have not been characterized previously, and possible mechanisms involved in the initial stages of LnA BH by rumen microbiota. The novel isomers identified in this thesis have been used to update the BH pathways for LnA (Figure 13) and LeA. These findings explain the appearance of several bioactive fatty acids in muscle and milk that influence the nutritional value of ruminant-derived foods.

1. Incubations of physiological amounts of LeA or LnA with bovine rumen fluid confirms that the BH pathway of LeA and LnA metabolism proceeds via isomerization to yield isomers of *cis*-9,*trans*-11 CLA or *trans*-10,*cis*-12 CLA formed from LeA and *cis*-9,*trans*-11,*cis*-15 CLnA formed from LnA. Increases in the doses of LeA or LnA increased the accumulation of these isomers. *Cis*-9,*trans*-11,*cis*-15 CLnA and other geometric isomers of Δ9,11,15 18:3 were formed from LnA via mechanism that appears identical to that responsible for the conversion of LeA to geometric isomers of Δ9,11 CLA. The investigations involving incubations of LnA with mixed rumen microbes diluted with D_2O suggest that the transformation of LnA to Δ9,11,15 CLnA is initiated by hydrogen extraction on C-11, formation of a radical intermediate and subsequent hydrogen transfer from water to C-13. The ^2H labels on C-9 and C-13 confirm that *trans*-11,*cis*-15 18:2 was formed from the reduction of *cis*-9,*trans*-11,*cis*-15 18:3.

2. Formation and characterization of the minor LnA intermediates provided evidence that besides the major BH pathway, BH of LnA may also proceed via several conjugated products, such as *trans*-9,*trans*-11,*cis*-13 CLnA, *trans*-9,*trans*-11,*cis*-15 CLnA, *trans*-11,*trans*-13 CLA, and *trans*-12,*trans*-14 CLA. Enrichment of the n+1 isotopomer of *trans*-9,*trans*-11,*cis*-13 CLnA increased over the course of incubations with D_2O, but was lower compared with incorporation of ^2H in Δ9,11,15 CLnA products. Nevertheless, increase in the abundance of the n+1 isotopomer above natural enrichment suggests that the conversion of *trans*-9,*trans*-11,*cis*-13 CLnA from LnA involves an exchange of H with water, by a mechanism that apparently differs from Δ9,11,15 CLnA formation.

3. Under the controlled conditions of this *in vitro* experiment, incubation of LeA or LnA with bovine rumen fluid resulted in the formation of so far uncharacterized isomers, such as *cis*-7,*cis*-12 18:2 and *cis*-8,*cis*-12 18:2 or *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3, respectively.

The accumulation of these NC 18:2 and NC 18:3 isomers indicates that metabolism of LeA and LnA can also proceed via migration of *cis*-9 double bond. Enrichment in isotopomers of *cis*-7,*cis*-12,*cis*-15 18:3 and *cis*-8,*cis*-12,*cis*-15 18:3 formed during incubations of LnA with D_2O did not differ from natural enrichment, indicating that formation of these products do not involve H exchange with water. Furthermore, increases in the initial doses of LeA or LnA increased the accumulation of these NC 18:2 and 18:3 isomers.

4. The extent of BH of LeA and LnA was confirmed to be time- and dose-dependent. Increases in the doses of LeA and LnA decreased the rate of BH and accumulation of 18:0. Reduction of 18:1 and 18:2 intermediates occurred at lower rates than the formation of CLA or NC 18:2 from LeA or 18:2 isomers from LnA and other 18:3 isomers. All transfer rates decreased with increases in LeA or LnA concentration.

5. Due to very low accumulation of Δ10,12,15 CLnA during incubation of LnA with rumen fluid it is not possible to confirm that the Δ10,12,15 CLnA isomer detected in this study is the missing product of LnA BH or to conclude about the possible mechanisms responsible. However, the amount of *trans*-10,*cis*-15 18:2 increased with increasing amount of added LnA. An increase in the n+2 isotopomer indicates that transformation of LnA to *trans*-10,*cis*-15 18:2 by rumen microbiota involves exchange of two H ions from water.

6. The main 18:1 isomers formed from LeA were *cis*-12 18:1, *trans*-10 18:1, and *trans*-11 18:1. Incubation of LnA with rumen digesta increased especially the content of *trans*-13/14 18:1. In all three experiments, the LeA or LnA was added to the incubation flasks as a suspension in aqueous Tween 80, which is a source of *cis*-9 18:1. Therefore, the 18:1 isomers, 18:0, and 10-O 18:0 accumulated in these studies (I-III) must be interpreted with caution.

7. The appearance of *cis*-12 18:1 and *cis*-12,*cis*-15 18:2 formed during incubations of LeA and LnA, respectively, with ruminal fluid could be considered as evidence of BH involving the direct reduction of *cis*-9 double bond, but it is also possible that *cis*-12 18:1 and *cis*-12,*cis*-15 18:2 originates from other BH intermediates.

8. The increases in the amount of several CLnA, CLA and NC 18:2 isomers formed during incubations of LnA with rumen fluid offers an explanation for the appearance of *cis*-9,*trans*-11,*trans*-15 CLnA, *trans*-9,*trans*-11,*cis*-13 CLnA, *trans*-11,*trans*-13 CLA, *trans*-12,*trans*-14 CLA, *trans*-10,*cis*-15 18:2, and *cis*-12,*cis*-15 18:2 observed in bovine muscle, adipose and milk fat.

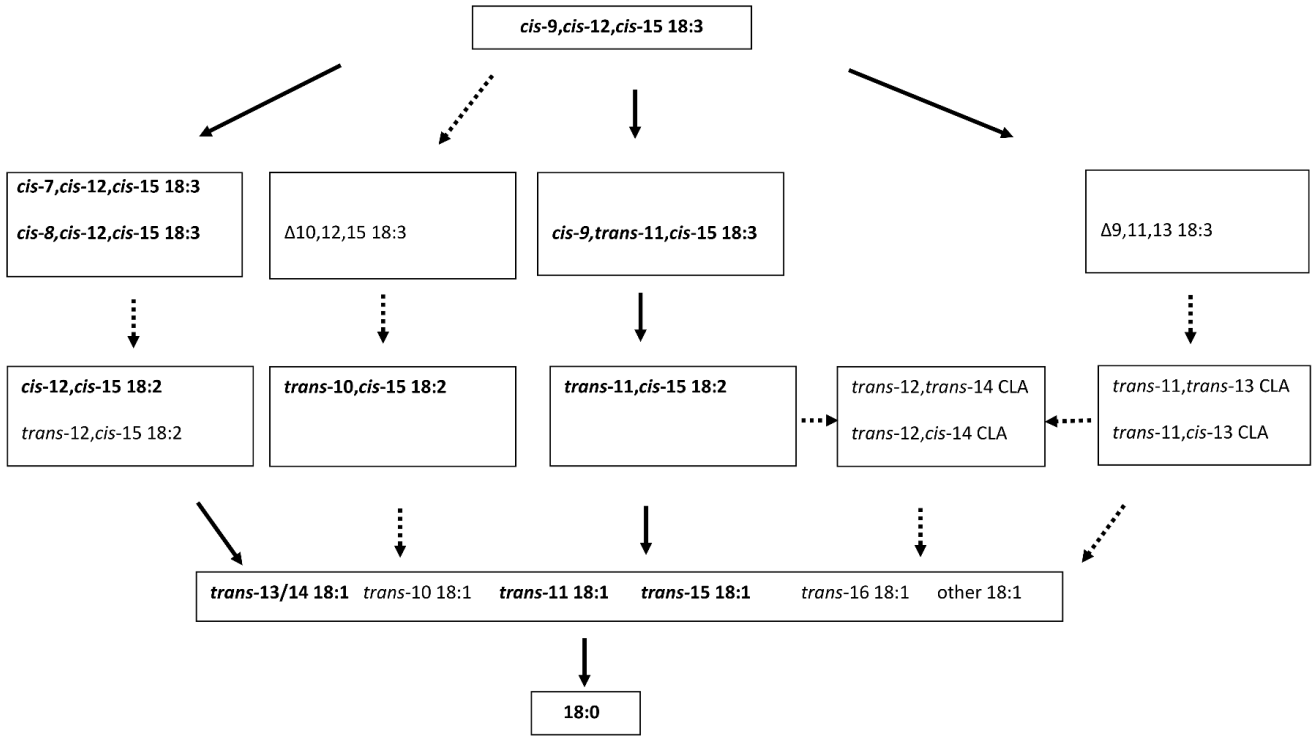

Figure 13 Summary of major and putative biohydrogenation pathways of alpha-linolenic acid (*cis*-9,*cis*-12,*cis*-15 18:3) during incubations with bovine rumen fluid *in vitro* (II, III). Dashed lines indicate putative pathways of alpha-linolenic acid biohydrogenation. Isomers in bold font indicate the production of major biohydrogenation intermediates. Isomers in regular font indicate the formation of minor metabolites formed during ruminal biohydrogenation of alpha-linolenic acid *in vitro*.

6 FUTURE RESEARCH

In future, it would be interesting to combine the GC-MS, GC-MS/MS, and NMR (nuclear magnetic resonance) techniques to identify the structure of BH products. Also, it would be interesting to compare the fatty acid profile after *in vitro* incubations of LeA and LnA by adding the fatty acids in the incubation flask dissolved in ethanol or emulsification through sonication rather than suspension in Tween 80. To confirm that the fatty acid isomers are formed during microbial BH, rather than chemical isomerisation and hydrogenation, it would be also good to have control samples without rumen fluid. Incubation of fatty acids with different substrates to create different fermentation environments would also be extremely interesting. The relationship between the concentrations of whole fatty acids could be studied by Pearson matrix, for example. Besides of the fatty acid profile, it would be of interest to examine the rumen methane emissions and to observe the possible changes in the microbial population. The *in vitro* method can be used to characterize the BH products formed during incubations of EPA and DHA with rumen fluid.

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